IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF A CLASS ON NON-SELECTIVE CATION CHANNELS IN *SACCHAROMYCES CEREVISIAE*

SCOTT ANTHONY WILLIAM CARTER

School of Agriculture, Food and Wine

The University of Adelaide

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1.   Introduction and review of literature

1.1. Introduction

Increasing salinisation of soils in Australia comes at an enormous cost to agricultural production. Presently there are 32 million ha of arable land in Australia that are affected by salt with the potential for 44 million ha to become affected by 2024 (FAO-AGL, 2000). This equates to a loss of agricultural production of approximately $130 million per annum (NAPSWQ, 2004). Minimising this loss is therefore of great importance to Australia.

Salt, in terms of soil salinity, consists of a mixture of ions dissolved in the soil solution. Of these ions, sodium (Na^+) predominates and is found mostly as NaCl. In many plant species, Na^+ is the cause of yield limitations associated with salinity through its influence on intra- and extra-cellular osmotic potential (Marin, 2004; Taji et al., 2004) and through competition with other ions necessary for plant function (Rubio et al., 1995). Further use of 'salt' in this thesis will refer to NaCl.

1.2. Overcoming soil salinity

To combat increasing levels of salinity stress in agricultural plant production a number of options have been suggested which include: 1) discarding or remediation of affected lands; 2) crop diversification towards more tolerant crops and 3) the directed conversion of current crops to those more tolerant to NaCl. Arable land in Australia is limited, thus discarding saline land is an unfeasible option in most cases.

Remediation is possible but expensive and time consuming. The introduction of new Na^+ tolerant food crops is possible but few viable and or suitable choices presently exist. Thus the genetic improvement of existing crops to those with enhanced salinity tolerance remains one of the best remaining options to combat salinity.

1.3. Plant based strategies for coping with salt stress

Strategies plants primarily use to maintain yield in saline conditions are exclusion and tolerance. The exclusion strategy is based around the plant being able to discriminate ions as they reach the plants cellular interface prior to entry. Examples of this can be found in some durum wheat varieties (Davenport, 2005). On the other hand tolerance to salt allows the plant to keep yielding in high Na^+ conditions. This is achieved through minimising the detrimental osmotic effects and overcoming toxic ion influences of Na^+.

1.3.1. Na^+ exclusion

Initial Na^+ flux into plant roots is rapid and of high capacity, contributing markedly to cellular Na^+ concentrations reaching toxic levels. (Cheeseman, 1982; Essah et al., 2003; Demidchik and Tester, 2002c; Davenport, 1998). The simplest method of a plant avoiding Na^+ toxicity is to stop its passage into root cells in the first place. Evidence of such Na^+ exclusion exists in some tolerant plant species. In barley, varieties that are tolerant to high concentrations of NaCl took up less Na^+ from the growth medium than the susceptible lines (Flowers, 2001). Na^+ exclusion has also been shown to occur within the plant itself. Davenport et. al. (2005) has demonstrated that partitioning of Na^+ between the leaf sheath and leaf blade is more prevalent in durum wheat genotypes that show Na^+ tolerance. Consequently, there is a strong

correlation between Na$^+$ exclusion from leaves and increased seed yield in durum wheat (Husain, 2003; Munns, 2003). The efflux of Na$^+$ from plant roots is also an exclusion mechanism that may be an important factor in Na$^+$ exclusion. Efflux of toxic cations such as Na$^+$ is thought to impose an increased energetic load on the plant which may impact on the overall toxicity response observed (Wang et al., 2006) (Malagoli et al., 2008). Comparison of the flux characteristics of the salt-tolerant *Thellungiella halophila* to its relatively salt-sensitive relative *Arabidopsis thaliana* (Wang et al., 2006) shows *T. halophila* appears to limit Na$^+$ uptake and thus the energistic requirements of Na$^+$ efflux to manage cellular Na$^+$ toxicity. As discussed in more detail in further sections, the physiology of Na$^+$ flux into plant roots affected by salinity suggest there are low affinity, high capacity protein(s) which may catalyse this flux.

1.3.2.   Na$^+$ tolerance

1.3.2.1.   Osmotic adjustment

At high external Na$^+$ concentrations, approximately 150 mM and above, the osmotic influence of NaCl appears to overwhelm the toxicity effects of Na$^+$ (Husain, 2003). Some plants have developed specific strategies to deal with such osmotic stress. For example, it is well documented that plants can respond to the osmotic adjustment imposed by salinisation of the rhizosphere through both increased salt accumulation (i.e. maintenance of the Na$^+$ / K$^+$ ratio) as described below and through the production of compatible solutes (Marschner, 1995). This response is supported by microarray data comparing the glycophyte *Arabidopsis thaliana* with that of the halophyte *Thellungiella halophilia* (Taji et al., 2004). The most immediate differences between

genus's are the marked up-regulation of genes responsible for the production of compatible solutes or 'osmoprotectants' in the halophytic *Thellungiella* (Taji et al., 2004). Overall, the osmotic component of high external NaCl concentrations has a profound effect on plant fitness. This often manifests itself in the reduction of cell elongation in the shoots and roots (Munns, 2008).

1.3.2.2. Maintaining Na^+ / K^+ ratios

There is a toxicity effect associated with Na^+ that is separate from osmotic influences. The effect is through competition with other cations, such as K^+, in biochemical interactions. For example, high concentrations of Na^+ decrease the uptake of $^{86}Rb^+$, an ion commonly used to trace the movements of K^+ (Epstein, 1962). The Na^+ / K^+ ratio indicates both how well the plant can nullify the competitive effects of Na^+ over K^+ in cellular reactions and how successfully it can minimise the uptake of Na^+ through internal osmotic and charge adjustment. The lower the Na^+ / K^+ ratio, the better the cell is considered to be at surviving elevated Na^+ levels (Munns, 2008). The rationale behind the Na^+ / K^+ ratio in terms of its use as a measure of Na^+ tolerance is still, however, a topic of debate (Kronzucker et al., 2008).

1.4.   Proteins that influence Na+ flux in plant cells

Given the importance of Na^+ flux across membranes in terms of salinity tolerance, it is not surprising that a great deal of work has been put into identifying and characterising membrane bound proteins that allow this flux to occur. Numerous proteins that influence cellular Na^+ concentrations *in planta* have been identified and their characteristics investigated. They include a Na^+ ATPase pump (Benito and Rodriguez-Navarro, 2003), the Na^+ / H^+ exchanger NHX1 in *Arabidopsis* (Gaxiola et

al., 1999), the low affinity cation transporter LCT1 (Schachtman, 1997), a Na^+ / K^+ co-transporter HKT1 (Schachtman, 1994; Rubio et al., 1995), the high affinity K^+ transporter HAK1 (Santa-Maria et al., 1997) and SOS1 (Halfter, 2000), a Na^+ / H^+ antiporter, and the group known as the <u>n</u>on <u>s</u>elective <u>c</u>ation <u>c</u>hannels (NSCCs) (Demidchik, 2002a).

1.4.1. Na^+ ATPase pump

The halophytic moss *Physcomitrella patens* possesses a Na^+ - ATPase Na^+ efflux pump that allows it to actively remove Na^+ from within its cells (Benito and Rodriguez-Navarro, 2003). Fungi such as *Saccharomyces cerevisiae* have also been shown to possess a similar pump, in this case termed ScENA1 (Haro et al., 1991). Such a system has no homologue in higher plants, however a similar system would be advantageous and could be considered as a viable alternative to remove accumulated Na^+ in plant cells.

1.4.2. Na^+ / H^+ Exchanger NHX1

The ability of a plant to sequester Na^+ in the vacuole is done in part by the action of Na^+ / H^+ exchangers. An example of this type of exchanger is the *Arabidopsis AtNHX1*. It was discovered through its sequence homology with the yeast *ScNHX1* (Nass et al., 1997). The effect of this gene was first shown in a study describing the effect it had in conferring salt tolerance when expressed in *S. cerevisiae* (Gaxiola et al., 1999). As exchangers such as NHX1 require a proton gradient across the tonoplast, the action of vacuolar H^+-pyrophosphatases have also been shown to influence Na^+ tolerance in plants.(Apse et al., 1999; Brini et al., 2007). Manipulation

of *NHX1*-like genes are useful in improving salt tolerance and possibly offer a path by which plants can be used to remediate salt affected soils.

1.4.3. The low affinity cation transporter, LCT1

Discovered by screening a wheat cDNA library in a *S. cerevisiae* K^+ transporter null mutant, LCT1 has been shown to facilitate the flux of Na^+ into cells in a low affinity manner (Schachtman, 1997). LCT1 has been shown to induce hypersensitivity to Na^+ when expressed in yeast (Amtmann et al., 2001) and also mediates Cd^+ and Ca^{2+} uptake (Clemens et al., 1998). While this protein has been shown to take up Na^+ in a low affinity manner, Ca^{2+} does not inhibit the Na^+ influx, in contrast to physiological observations in planta. This transporter could contribute to total plant Na^+ flux, but, given its Ca^{2+}-insensitivity, it seems unlikely to be the viNSCC, considered the major contributor.

1.4.4. The high affinity Na^+ / K^+ transporters, the HKT family

The HKT1 gene was initially identified in wheat due to its properties as a high affinity K^+ transporter (Schachtman, 1994). HKT1 was subsequently shown to mediate both H^+ / K^+ and Na^+ / K^+ co-transport when expressed in yeast (Rubio et al., 1995). HKT1 was subsequently shown to influence Na^+ homeostasis in Arabidopsis and influencing K^+ nutritional status as a consequence (Rus et al., 2004). Other members of the HKT family have been implicated with Na^+ tolerance in planta, such as Na^+ exclusion phenotypes observed in durum wheat (Byrt et al., 2007). The number of HKT family members and their diversity across species suggest they may have a diverse range Na^+ or K^+ related function in plants (Platten et al., 2006).

1.4.5. The Na^+ / K^+ co-transporter HAK1

The HAK1 gene family in plants seem to play a role in cellular Na^+ and K^+ homeostasis in plants. In barley, Na^+ was shown to inhibit Rb^+ uptake at millimolar concentrations but not at micromolar concentrations (Santa-Maria et al., 1997). The protein *HvHAK1* has thus been described as capable of low affinity Na^+ uptake within plants (Horie and Schroeder, 2004). The kinetics of this uptake does not exhibit classical unsaturable uptake characteristics (Figure 1-1), nor has Na^+ / K^+ uptake been observed to occur in roots (Maathuis et al., 1996). These properties make it unlikely that the HAK1-like proteins are responsible for the high Na^+ flux observed in plant roots.

1.4.6. The SOS1 Na^+ / H^+ antiporter

Named because of the salt overly sensitive phenotype its mutation imparted, the SOS1 gene was first mapped to chromosome 2 of *Arabidopsis* through the use of a root bending assay (Wu et al., 1996). The protein this gene encodes for was later deduced to be a Na^+ / H^+ antiporter (Shi et al., 2000). Like the *NHX1* antiporter, *SOS1* shows promise in the development of Na^+ tolerant plants.

1.4.7. The Non-Selective Cation Channels (NSCCs)

Non-selective cation channels (NSCCs) facilitate the movement of a range of cations including: K^+, NH_4^+, Ca^{2+} and Na^+ (Demidchik, 2002a). NSCCs can be classified as hyperpolarisation activated cation channels, depolarisation activated cation channels,

Ca^{2+} activated cation channels, voltage independent NSCCs, glutamate activated NSCC's and cyclic-nucleotide gated channels (CNGC's).

1.5. Candidate proteins that facilitate non-selective cation flux

There are a number of potential pathways that could account for the rapid flow of Na^+ in plants. These include a number of proteins known from physiological data as well as those with a known molecular identity such as the glutamate activated NSCCs and the cyclic nucleotide channels.

1.5.1. Glutamate activated NSCCs

Glutamate like receptors (GLR's), form a large family within plants. GLR's were first identified through their homology with animal glutamate receptors (Lam, 1998), where the compound glutamate acts to gate cation channels that span cellular membranes. GLR's have changed little since plants and animals diverged on their evolutionary pathways (Chiu et al., 1999), suggesting they perform important functions in living organisms. There are 20 members and 1 pseudo-gene of the plant GLR family identified in *Arabidopsis* (Lacombe et al., 2001), consisting of 3 sub-groups, classed according to the proportion of sequence identity they share. Elucidating a glutamate-induced response from plant GLR's has been unexpectedly difficult. The application of glutamate induces calcium fluxes in the cytosol of *Arabidopsis* (Dennison and Spalding, 2000) and the application of glutamate to *Arabidopsis* root protoplasts increases the flux of Na^+ and Ca^{2+} across the membrane

(Demidchick, 2004). However, when individual plant GLRs have been studied they behave as non-selective cation channels but do not show a notable response to glutamate (Kim et al., 2001; Davenport, 2002).

1.5.2. Cyclic Nucleotide Gated Channels

In animals, cation channels that are gated by cyclic nucleotides such as cAMP and cGMP have been widely described. Examples include the olfactory signalling process and the flux of K^+ and Na^+ as a response to light in the rods and cones of ocular systems (Stryer, 1986). Responses of cation channels to cyclic nucleotides were first reported in cultured carrot cells (Kurosaki, 1994) and have since been described in *Arabidopsis* (Maathuis and Sanders, 2001). Like plant GLRs, the molecular identity of plant cyclic nucleotide gated channels, (CNGCs) were also found by screening plant ESTs with conserved motifs found in animal CNGCs (Kohler et al., 1999; Leng et al., 1999). They allow the passage of mono-valent cations as well as Ca^{2+} and also seem to interact with calmodulin (Kohler et al., 1999; Kohler and Neuhaus, 2000).

1.5.3. Hyperpolarisation activated NSCCs

Hyperpolarisation activated NSCCs have been described in *Arabidopsis* root hairs (Very and Davies, 2000; Kiegle et al., 2000b) and tomato (Gelli, 1997). They allow the passage of both mono-valent and divalent cations but are blocked by trivalent cations (eg. La^{3+}, Al^{3+}). Such channels have been associated with apical root cell elongation in *Arabidopsis* (Kiegle et al., 2000b).

1.5.4. Depolarisation activated NSCCs

Depolarisation activated NSCC's have been described in the seed coat of *Phaseolus vulgaris* (Zhang, 2000), *Arabidopsis* (Pei et al., 1998), barley (de Boer A. H., 1997), rye (White, 1998, 1999a) and wheat (Pineros, 1997). They allow the passage of mono-valent cations, including NH_4^+, choline$^+$ and TEA^+. Some divalent cations, such as Ca^{2+}, were shown to pass through these channels but other divalent cations exhibit reversible blocking characteristics. The trivalent Gd^{3+} blocked the channel in a partially irreversible manner (White, 2000).

1.5.5. Ca^{2+} activated NSCCs

The Ca^{2+} activated NSCC's are not widely reported in plants (Elzenga, 1994; Bewell et al., 1999), despite being widely reported in animal systems (Teulon, 2000). As Ca^{2+} plays an important role in plant signalling, it is possible that Ca^{2+} activated NSCC(s) may also operate in plants.

1.5.6. Voltage insensitive NSCCs

The voltage insensitive NSCCs have been well characterised physiologically but are as yet also short of a molecular identity(s). Such properties are localisation in the plasma membrane, the absence of (or weak) voltage gating, being non-specific in terms of mono-valent cations and blocked by divalent cations such as Ca^{2+} and Mg^{2+}. However, White and Davenport (2002) have reported a voltage insensitive NSCC that is permeable to Ca^{2+} in wheat roots. Voltage insensitive NSCCs have been found in rye (White, 1992), wheat (Buschmann et al., 2000; Davenport and Tester, 2000), *Arabidopsis* (Demidchik and Tester, 2002c), soybean (Tyerman et al., 1995) and

barley (Amtmann et al., 1997). It has been suggested that this group of NSCC's may provide the majority of Na^+ influx into a cell (White, 1992; Amtmann, 1999; Tyerman, 1999; White, 1999a) which encourages further investigation of Na^+ flux through this class of channel.

The physiological role of voltage insensitive NSCCs is not limited to Na^+ flux alone. The movement of large amounts of cationic charge that can occur through such channels suggest possible role in the maintenance of intracellular charge ratios or turgor (Tyerman, 1999). The maintenance of osmolarity could also rely on such channels for rapid response to altered turgor requirements. By allowing the rapid flux of K^+, turgor could be adjusted quickly through the associated change in osmotic potential. A voltage insensitive NSCC-like channel would be very important in the maintenance of cellular K^+ concentrations.

The low affinity movement of NH_4^+ across plant membranes occurs through a currently unknown mechanism. There have been reports of NH_4^+ flux through voltage insensitive NSCCs (White, 1996; Davenport and Tester, 2000). This has resulted in the suggestion that voltage insensitive NSCCs would make good candidates for low affinity ammonium transport throughout the plant (Tyerman et al., 1995; Davenport, 1998; Amtmann, 1999; Kronzucker, 2001). When the potential physiological properties of voltage insensitive NSCCs are considered with respect to Na^+, K^+ and NH_4^+, the need for their further characterisation at a molecular level is highly desirable.

1.6. Enhancing Na$^+$ exclusion characteristics

Na$^+$ flux in the low affinity range (known as the <u>l</u>ow <u>a</u>ffinity <u>u</u>ptake <u>s</u>ystem, LATS) is considered the most influential on initial Na$^+$ entry into plants (Epstein, 1965; Davenport, 1998; Davenport, 2005; Munns, 2008). Some proteins active in the HATS range, such as the HKTs, have been shown to improve salt tolerance (Munns, 2008) although these are not thought to be the major pathway for initial Na$^+$ entry. Analysis of ion flux in root cells strongly suggests viNSCCs are the likely candidates for this flux (Davenport and Tester, 2000; Demidchik and Tester, 2002c). The viNSCC class of channels are distinguished by their ability to catalyse the flux of mono-valent cations in a non-selective manner and that they are also sensitive to pH and the presence of divalent cations such as Ca^{2+}. Interestingly, adding Ca^{2+} in the form of gypsum can exert an ameliorative effect on Na$^+$ toxicity in soil grown plants (Marschner, 1995). Electrophysiological investigations of various plant and yeast cellular membranes have demonstrated that Na$^+$ channel activity (including viNSCC channel activities) are inhibited by exogenous Ca^{2+} (White, 1992; Tikhonova et al., 1997; Tyerman et al., 1997; Buschmann et al., 2000; Davenport and Tester, 2000; Demidchik, 2002a). Breeding programs directed at improving salinity tolerance may benefit greatly if the physiology of this channel could directly monitored for variation and that selections be made based upon reduced Na$^+$ flux through this channel. To achieve this, the elucidation of the molecular identity of the viNSCC is necessary.

1.7. Finding candidate NSCCs

Patch clamp analysis by Bihler et al (1998) of wild type *S. cerevisiae* spheroplasts (strain Y588) revealed the presence of non-selective cation channels, which they

called NSC1. Subsequently, further work by Bihler et. al. (2002) in the strain CY 152/162 characterised the nature of the channel current which was shown to facilitate Na^+, K^+ and NH_4^+ flux rapidly across the yeast spheroplast membrane. It was also shown to be pH and Ca^{2+} sensitive, where it was blocked by high concentrations of Ca^{2+} (10 mM) and pH of 4.0. The channel was shown to be open at $[Ca^{2+}] = 0.2$ mM and at pH 7.0 (Figures 1-2, 1-3 and 1-4). This channel shares many characteristics with the viNSCCs observed in plants. Knowledge of the physiology of this protein coupled with the powerful techniques available for use with yeast systems could potentially be used to elucidate the identity of NSC1. As NSC1 shares properties with viNSCCs in other systems there may be a degree of sequence conservation between these proteins. The determination of the sequence of NSC1 may allow viNSCCs in other systems to be identified through sequence similarity.

1.7.1. The strain 26972c as a NSC1 null mutant

Whilst examining the electrophysiology of NH_4^+ transport in the *S. cerevisiae* strain 26972c, Kaiser et. al. (1998) demonstrated there was no inward K^+ flux as described by Bihler et. al. (2002) in CY 152/162 (Figure 1-5). This suggests that the NSC1 protein is not active in the strain 26972c. Examination of the history of this strain offers an explanation as to why this may be the case.

The strain 26972c was created by EMS mutagenesis of the wild type yeast strain $\sum$1278b. The mutants created through the addition of EMS were screened according to their ability to survive in the presence of 100 mM of the toxic NH_4^+ analogue methylammonium (MA^+). By doing this, Dubois and Grenson (1979) selected the mutant strain 26972c that was deficient in functional versions of its three native high

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affinity NH_4^+ transporters, known as Meps. The strain 26972c has a point mutation within Mep1, physical deletion of Mep2 and trans inhibition of Mep3 through the mutation in Mep1. As NSC1 was shown to allow the flux of NH_4^+ (Figure 1-3) (Bihler, 2002) and plant viNSCCs have been shown to catalyse the flux of MA^+ (Davenport, 1998) (Figure 1-6), it is likely that NSC1 will also allow the flux of MA^+. Therefore, it is also likely that any strain that is selected for its inability to take up MA^+, and therefore survive on media containing MA^+, will be missing a functional NSC1 protein. The lack of NSC1 K^+ flux and the screening process that created 26972c suggests this strain may also be a NSC1 null mutant (Figure 1-5).

1.8. Conclusion

Improving salt tolerance in crop plants will be assisted through the use of physiological knowledge concerning the passage of Na^+ into and throughout the plant. Uptake experiments have determined that Na^+ rapidly enters the roots and is translocated throughout the plant in a similarly fast manner (Essah et al., 2003) (Figure 1-7). The logical conclusion to be drawn from this is that a plant that can reduce such flux into the plant or its translocation could potentially improve its salt tolerance. The electrophysiological evidence that has been accumulated with respect to Na^+ flux shows many different pathways exist that facilitate Na^+ flux throughout the plant. The most likely pathway to catalyse such rapid Na^+ flux is through the voltage insensitive NSCCs (viNSCCs). No molecular identity has been assigned to any plant channel in this class at present. Having a molecular identity would both increase the depth of knowledge on Na^+ uptake characteristics in plants and give a definite and accurate phenotype for plant breeders to use in salt tolerance based breeding programs.

The elucidation of a molecular identity for voltage insensitive NSCCs in plants is difficult due to the universality of this channel type throughout living organisms. There are no known *in planta* voltage insensitive NSCC null mutants that can be used for genomic comparison. There is as previously discussed, a strain in yeast that appears to be deficient in this protein, 26972c. In this thesis, I have utilised the unique cation transport properties of 26972c to help identify phenotypes associated with putative NSCC's via the use of cDNA and transposon library screens as well as a comparative bioinformatic approach.

Some putative non-selective cation channels in plants that have been given molecular identities, the GLRs and CNGCs, were first discovered because of the similarities their sequences share with proteins in other organisms. As viNSCCs share very similar properties across yeast (Bihler, 1998, 2002), plants (Davenport, 1998; Davenport and Tester, 2000; Demidchik et al., 2002b; Demidchik and Tester, 2002c) and animals (Hagiwara et al., 1992), finding the molecular identity of yeast voltage insensitive NSCCs could potentially lead to the identification of plant voltage insensitive NSCCs in other systems.

Figure 1-1: Inhibition of Rb^+ uptake by HKT1 in the presence of increasing concentrations of Na^+ and K .

Both K^+ and Na^+ uptake competitively inhibit Rb^+ uptake in a strain of *Saccharomyces cerevisiae* that is expressing *HvHKT1*, suggesting their flux is competitively catalysed through the same process. The strain is deficient in the native high affinity K^+ transporters *TRK1* and *TRK2*. (From Santa-Maria et. al., 1997, Figure 4).
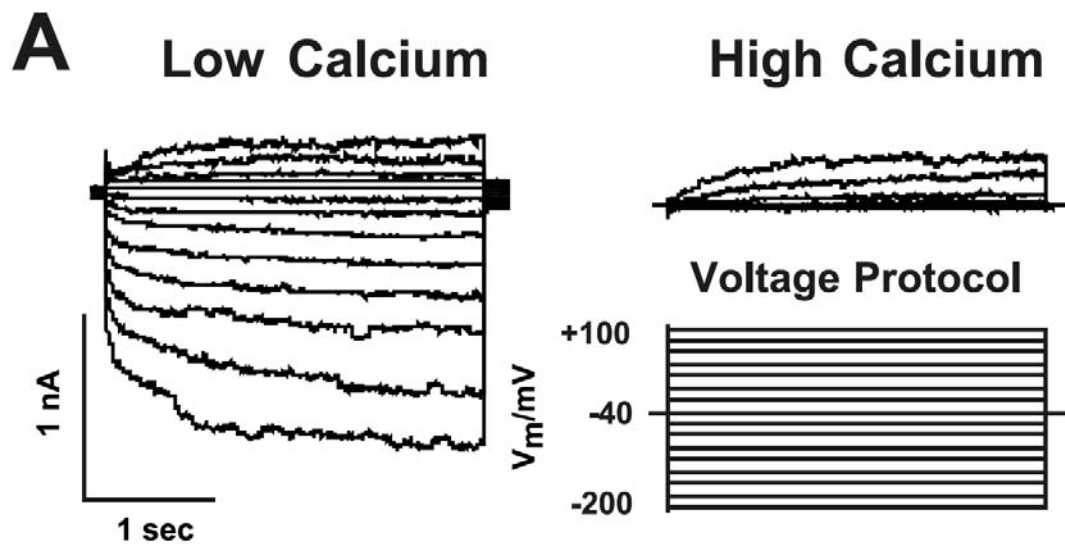
Figure 1-2: The effect of Ca^{2+} on NSC1 activity

The trace below represents the flow of K^+ through the *Saccharomyces cerevisiae* channel NSC1 into yeast spheroplasts. At low Ca^{2+} (0.2 mM) the channel is open and allows for K^+ flux. With the presence of high Ca^{2+} (10 mM) in the bathing solution, the channel is blocked and there is no inward movement of K^+. Taken from Bihler et. al., 2002, in the *S. cerevisiae* strain CY 152/162.

A

150 mM K$^+$ (Control)

150 mM NH$_4^+$

500 pA

1 sec
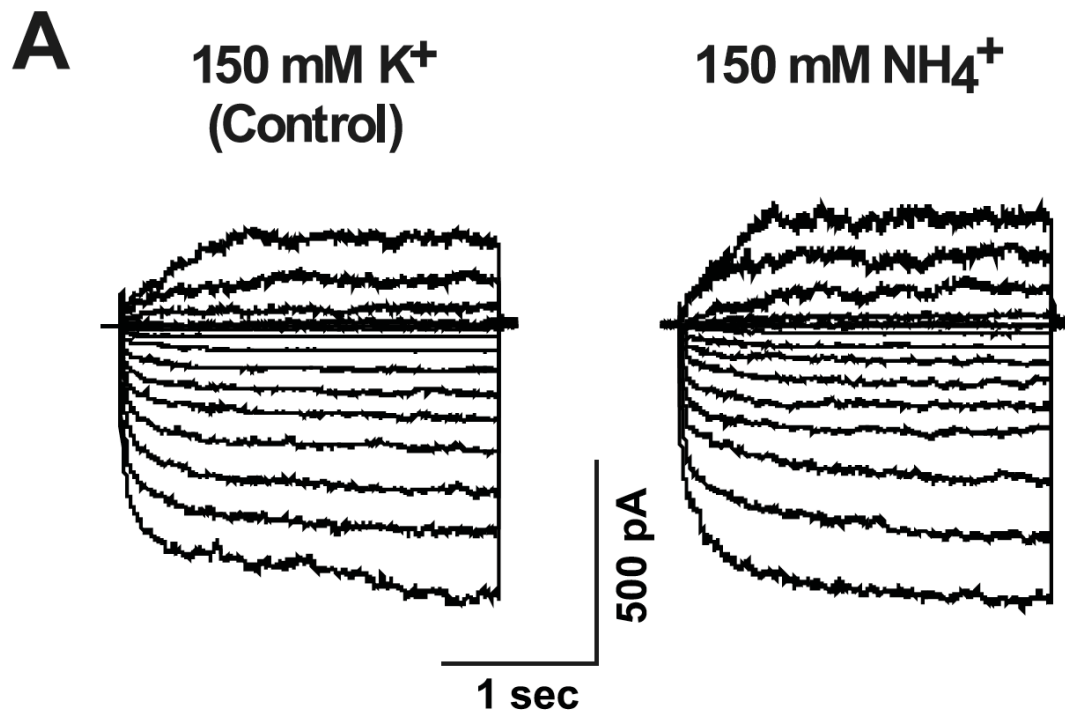
Figure 1-3: The flux of NH$_4^+$ through NSC1

These traces illustrate how NH$_4^+$ flux through the NSC1 channel has very similar characteristics to that of K$^+$ (Figure 1-2). Data taken from Bihler et. al., 2002, in the *S. cerevisiae* strain CY 152/162.

A   pH 7.5   pH 4.0

250 pA

1 sec
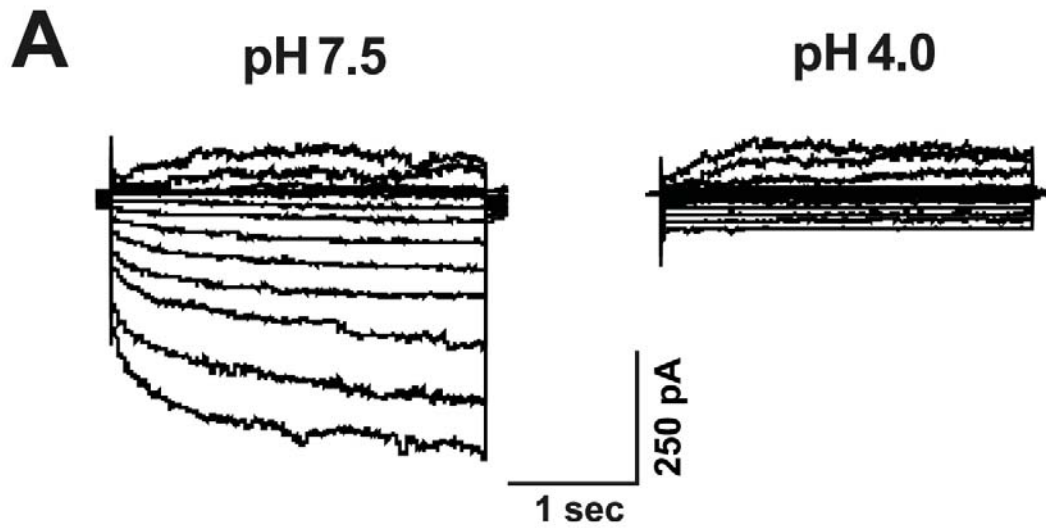
Figure 1-4: The effect of pH on NSC1

These traces show that at pH 7 NSC1 is open, allowing for the inward flux of K^+ to occur. At pH 4.0, the channel is blocked and K^+ inward flux does not occur. Data taken from Bihler et. al., 2002, in the *S. cerevisiae* strain CY 152/162.

| A | *S. cerevisiae* strain 26972c | B | *S. cerevisiae* strain CY 152/162 |

From (Kaiser et al., 1998)) Figure 6

From Bihler et. al. (2002), Figure 2
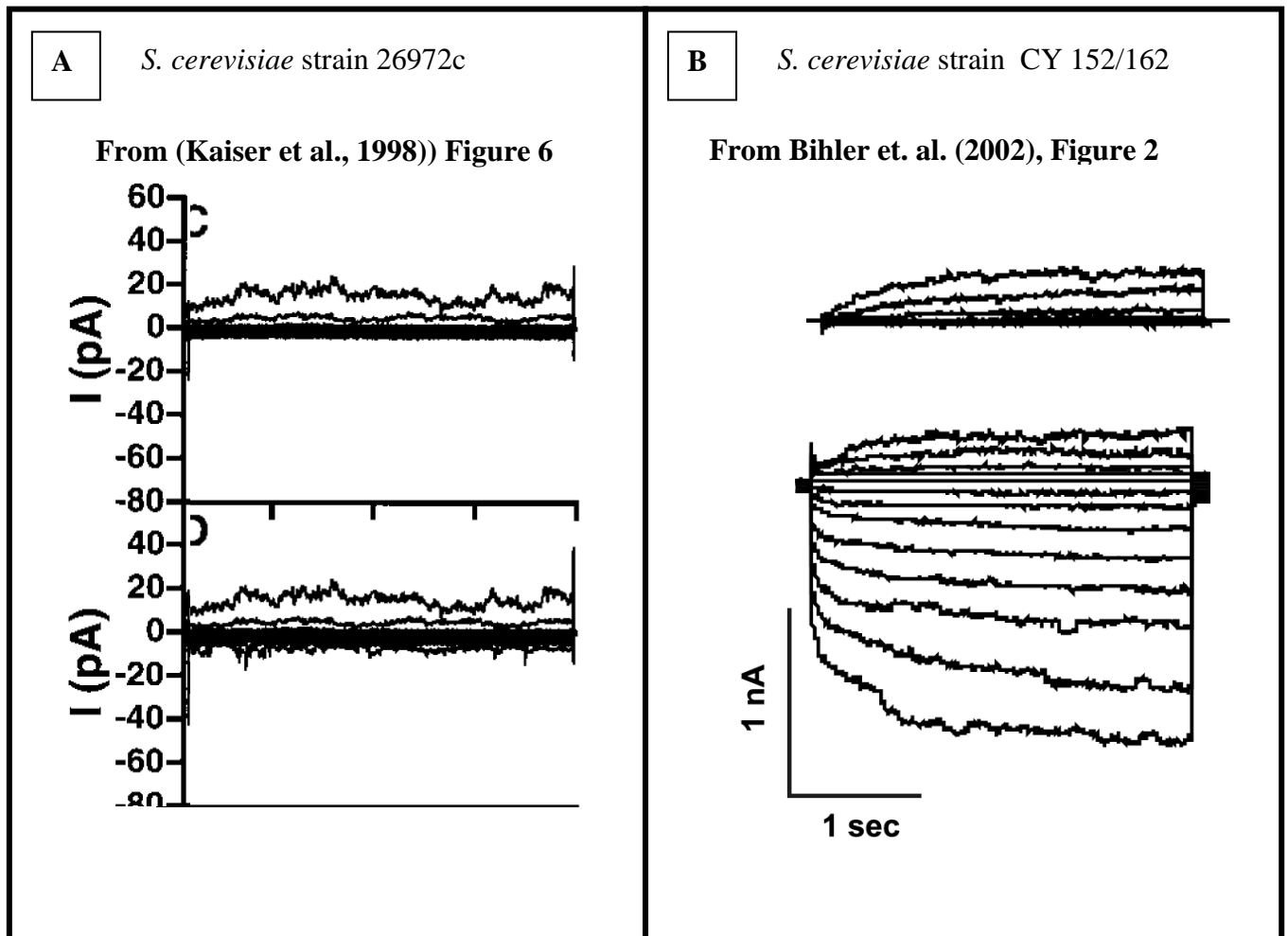
Figure 1-5: Comparison of 26972c and CY 152/162

The above figures show the differences in K^+ flux between the strains 26972c and CY 152/162. Both sets of patch clamping were performed under very similar conditions in terms of bathing solutions. The traces for 26972c (A) clearly show that there is no inward trace caused by the movement of K^+ compared to the clear inward trace shown in CY 152/162 (B). This leads to suspicions that 26972c is missing its native NSC1 channel.

Figure 1-6: Comparison of currents through the viNSCC of wheat protoplasts induced by the flux of Na^+ or MA^+.

I/V relationships between the viNSCC current in wheat protoplasts for Na^+ (open circles), MA^+ (open diamonds) and equimolar Na^+ / MA^+ (closed circles). Cation concentrations are indicated by the number annotations on the left, units in mM. Taken from Davenport (1998).

Taken from Essah et. al. (2003)

Figure 1-7: Rapid accumulation of Na$^+$ into *Arabidopsis thaliana* roots.

Net Accumulation of Na$^+$ in *Arabidopsis* roots after immersion in a solution containing 200 mM NaCl and 0.2 mM Ca^{2+}. (A) Accumulation of Na+ over time. (B) Accumulation in (A) over the initial 2 minutes. The data indicates rapid Na$^+$ flux occurs in the initial two minutes of exposure to Na$^+$.

2. Development of a Saccharomyces cerevisiae based screen for NSCCs

2.1. Introduction

Physiological observations in plants, such as the rapid uptake of Na^+ from the soil solution and its translocation into the shoots, have been associated with Na^+ sensitivity (Essah et al., 2003; Davenport, 2005). Such rapid, high capacity flux across plant membranes is widely thought to occur via cation channels. Physiological investigations have revealed evidence of facilitated low affinity cation flux across biological membranes in both yeast and plant cells (Tyerman et al., 1995; Bihler, 1998; Demidchik, 2002a). The class of NSCC described as the weakly voltage dependent or the voltage independent NSCC (viNSCC) (Davenport and Tester, 2000) is of particular interest for salinity tolerance in plants as it appears to facilitate the rapid, high capacity flow of Na^+. Properties such as the non-selective nature of mono-valent cation flux, pH and Ca^{2+} sensitivity, which are attributed to these channels as described in *Saccharomyces cerevisiae* and in plants, show a degree of commonality between systems (Bihler, 1998; Davenport and Tester, 2000; Bihler, 2002). It must be noted, however, that pH and Ca^{2+} concentrations are involved in many aspects of cell biochemistry and the physiology. Care must be taken when interpreting data influenced by these factors so the overall functions of the system being investigated are not ignored.

No molecular identity had been determined for the viNSCC until this study. This is possibly due to the nature of the flux they facilitate which is difficult to physically quantify due to its bidirectional activity, flux capacity, gating, and selectivity) in high throughput genetic screens using heterologous expression systems. Alternatively

over-expression of viNSCC's in heterologous cells may impart toxicity phenotypes that limit their detection. Phenotypes associated with this class of proteins are characterised by their distinct electrophysiological profiles (Hagiwara et al., 1992; Tyerman et al., 1995; Bihler, 1998; Davenport, 1998; Tyerman, 1999; Davenport and Tester, 2000; Bihler, 2002; Demidchik, 2002a; Demidchik and Tester, 2002c). Therefore, the ability to identify the presence or absence of these activities will be essential to ultimately identify putative genes encoding viNSCC's.

Bihler et al. (1998 & 2002) described a viNSCC in *S. cerevisiae*, called NSC1. NSC1 is non-selective to mono-valent cations, catalysing K^+ and NH_4^+ flux in similar fashions. NSC1 is also sensitive to Ca^{2+} with 10 mM Ca^{2+} abolishing inward current due to K^+ whereas 0.2 mM Ca^{2+} does not. Sensitivity to pH was also described, with the channel in its 'open' state at a pH of 7.5 but closed at pH 4.5.

2.1.1. A potential viNSCC null mutant in yeast

The initial characterisation of NH_4^+ transport in *S. cerevisiae* utilised ammonium transport mutants derived from EMS mutagenesis of the wild type yeast strain $\sum$1278b. Mutants were selected based on their capacity to grow on media containing 100 mM of the toxic NH_4^+ analogue methylammonium (MA^+). A number of ammonium transport mutants were identified and a few, including the haploid strain 26972c, demonstrated strong resistance to MA^+ and loss of NH_4^+ transport capacity (Dubois, 1979). 26972c showed impaired MA^+ influx capability, it did not accumulate MA^+ to toxic levels. Investigations of this strain have since revealed it has non-functional high affinity ammonium transporters (MEPs) (Marini et al., 2000) suggesting MA^+ toxicity is due to the action of MEPs alone. The MEPs are, however,

not the only pathway for MA^+ influx into yeast cells. Two distinct systems exist for MA^+ uptake. One operating in the high affinity (0 – approximately 5 - 8mM) known as the High Affinity Uptake System (HATS) and the other at low affinity (5 - 8mM and above) concentrations, known as the Low Affinity Uptake System (LATS) (Dubois, 1979). As the concentration of MA^+ that was used in the selection of 26972c is well into the low affinity range (100 mM), it is a reasonable assumption that a mutation impairing LATS may also have been selected for, additional to the well characterised MEP mutations (Marini, 1994; Marini et al., 2000).

Kaiser et al. (1998) patch clamped the *S. cerevisiae* strain 26972c under conditions very similar to those employed by Bihler et. al (1998) in the characterisation of NSC1. Comparing the traces found in Bihler (2002) with Kaiser et. al. (1998) , no evidence of an active NSC1 was detected in 26972c (summarised in Figure 1-5). This combined with the LATS range MA^+ concentrations used to select 26972c suggest NSC1 may be inactive in this strain.

The physiological similarities between plant and yeast viNSCCs (Bihler, 1998; Davenport and Tester, 2000; Bihler, 2002) would suggest MA^+ flux through a yeast viNSCC would also occur through a plant viNSCC as shown by Davenport (1998). These similarities allowed the development of a MA^+ based yeast screen to identify both plant and yeast viNSCCs.

2.1.2. Development of a screen for viNSCCs

A series of experiments was designed to investigate the use of yeast Mep mutants as heterologous screening tools for discovering viNSCCs. A mutation in the MA^+

resistant 26972c effectively disrupts the function of all three Meps. A point mutation in the C terminus of Mep1 both abolishes its activity but also acts to trans inhibit the activity of the remaining functional Mep3, while Mep2 is completely deleted from the genome. To avoid any possible reactivation of Mep3 and subsequent false positives in the screen the strain 26972c2, derived from 26972c through the deletion of Mep3 by homologous recombination, was used (B. Kaiser, unpublished results). An alternative MA$^+$ resistant strain, 31019b (Marini et al., 1997) was also used, 31019b, which is isogenic to the WT $\sum$1278b but with all three Meps deleted through homologous recombination. Thus both strains share the same parent and have inactive Meps, where 31019b has a WT background and 26972c2 has an EMS mutagenised background. It was hypothesised the 26972c2 strain has also lost the activity of the functional yeast NSC1.

2.2. Results

2.2.1. Differences in MA$^+$ accumulation in ∑1278b, 26972c, 26972c2 and 31019b

Yeast strains 31019b, 26972c, 26972c2 and ∑1278b were grown as described in materials and methods (Section 2.4.1) to investigate their MA$^+$ accumulation phenotypes (Figure 2-1). The *ura-* selection phenotype was complimented in 31019b, 26972c and 26972c2 via transformation with the empty *E. coli* / yeast binary vector, pYES3 (Smith et al., 1995). ∑1278b did not grow on any of the media containing MA$^+$ although dead cells left a 'watermark' on the media. 31019b, 26972c and 26972c2 showed improved survival in the presence of MA$^+$ compared to ∑1278b. On media with 10 mM Ca^{2+} and / or pH 6.5 and below, 31019b cells grew in a manner identical to 26972c and 26972c2 (Figure 2-1A). A difference was seen on media with 0.2 mM Ca^{2+} at pH 7.0 and greater. Under these conditions, 31019b grew noticeably less than 26972c and 26972c2 (Figure 2-1, B). The differences between 31019b and the two 26972c based strains in terms of the influence of Ca^{2+} and pH mirrors the conditions shown to influence NSC1 (Bihler, 1998, 2002) (section 1.5.6) suggesting 31019b has a functional NSC1, allowing greater MA$^+$ accumulation under open channel whereas 26972c and 26972c2 do not.

2.2.2. Screening cDNA libraries for viNSCCs using the phenotypic differences between 31019b and 26972c2

A screen to identify putative viNSCCs from cDNA libraries was developed based on the differences in growth patterns between 31019b and 26972c2 on media containing MA^+ and varied levels of $[Ca^{2+}]$ and or pH. The screen consisted of a positive and a negative approach. The positive screen involved selecting for plant cDNAs, that when expressed in 26972c2 would increase sensitivity to MA^+ on media at pH 7 containing low Ca^{2+} (0.2 mM), conditions where 26972c2 is natively MA^+ tolerant. In contrast, the negative screen used a transposon insertion library (Kumar et al., 2004) to first mutagenise 31019b to lose native viNSCC activity. Individual cells were to be selected based on improved growth on MA^+ media at pH 7 containing low Ca^{2+} (0.2 mM). Identified colonies were verified for insertional events using inverse PCR.

2.2.2.1. Positive screen using a *Glycine max* nodule cDNA library

S. cerevisiae strain 26972c2 was transformed with a *Glycine max* cDNA library ligated into pYES3 (Kaiser et al., 1998) and grown in the presence of 100 mM MA^+ with either normal (10 mM) or reduced (0.2 mM) Ca^{2+} pH 7.0. Forty putative colonies were selected through this initial screen (Figure 2-2), which were then re-screened on similar media. Only five colonies showed similar phenotypes (retention of the Ca^{2+} sensitive phenotype when grown in the presence of 100 mM MA^+) In the second re-screen (Figure 2-3) while the remaining were deemed false positives.

The five candidate genes selected were allocated an identity based upon their position in the 384 well plates (Table 2-1). Growth phenotypes were then verified again on

solid yeast minimal media containing either: a) 100 mM MA$^+$, 0.1% L-proline, 2% D-galactose, 0.2 mM Ca^{2+}, pH 7.0; b) 0.1% L-proline, 2% D-galactose, pH 7.0 10 mM Ca^{2+} or c) yeast nitrogen base (BD Biosciences) + 2% D-glucose as a loading control (Figure 2-3). P2 B7.1 showed increased overall sensitivity to MA$^+$ relative to the pYES3 controls. Both P10 A10.8 and P9 E11.5 showed a pH and Ca^{2+} response. P2 A2.6 and P7 B8.22 both display improved survival on MA$^+$ media at both 0.2 mM Ca^{2+} and 10 mM Ca^{2+} (Figure 2-3). Identities of each cDNA were determined (Section 2.4.2, Table 2-1 and Figure 2-4). P2 B7.1 and P10 A10.8 aligned to *G. max* leghaemoglobin 1 and c-2 proteins respectively (GI:18595 and GI:169994). P10 A10.8 aligned to a *Glycine max* Proline Rich Protein (GI:170048) and P9 E11.5 to a *G. max* peroxidase precursor protein GMIPER1 (GI:2245682). P7 B8.22 was shown to express a MYB transcription factor, MYB138 (GI:110931719).

2.2.2.2. Negative screen using a transposon insertion *Saccharomyces cerevisiae* cDNA library.

A mTn transposon insertion library (Kumar et al., 2004) was transformed into the strain 31019b on two separate occasions. This allowed for approximately 10^6 transformation events to occur and subsequently be screened. 5 transposon insertion mutant clones displayed a phenotype in 31019b similar to that expected for a viNSCC knockout mutant when grown on media containing 100 mM MA$^+$, 0.2 mM Ca^{2+} pH 7.0 (Figure 2-5). The site of mTn insertion was determined through the use of inverse PCR (section 2.4.3). Unfortunately, all 5 of these mutants had transposons inserted in regions coding for subunits of the yeast ribosomal RNA complex (Table 2-2, Figure 2-6). All insertions but one occurred in the locus YDR418w (RPL12B) which is a

subunit of the 60s ribosomal complex and the other in RDN25-2, another component of 60s subunit of ribosomal RNA.

2.3.   Discussion

NSCCs have a significant influence on cell biochemistry by virtue of the high capacity non-selective nature of cation flux they facilitate. These proteins can be further classified according to phenotypes they exhibit. The viNSCC class is defined largely by their non selectivity towards mono-valent cations, the sensitivity of mono-valent cation flux to divalent cations and to pH and, to a lesser extent, the apparent insensitivity of cation flux to membrane potential (Hagiwara et al., 1992; White, 1992; Tyerman et al., 1995; White, 1996; Roberts, 1997; Bihler, 1998; Roberts et al., 1999; Davenport and Tester, 2000; Bihler, 2002; Zhang et al., 2002; Demidchik, 2002a). These properties of viNSCCs appear to be reasonably ubiquitous across the range of organisms they have been described in. The conservation of protein structural properties is likely to be the source of this conserved phenotype. The identification of a viNSCC protein in one organism may, through the use of bioinformatic resources and search algorithms, allow for other members of the group to be identified both within the same organism and between other organisms.

The *S. cerevisiae* system is a well studied and relatively easy to manipulate at a molecular level, making it an ideal tool for the identification of viNSCCs. NSC1 is an identified viNSCC in *S. cerevisiae* shown to catalyse K^+ and NH_4^+ flux in a low affinity manner in the strain CY152/162 and the wild type strain Y588 (Bihler, 1998, 2002). A putative NSC1 null mutant strain, 26972c (Kaiser et al., 1998), was used as part of a series of screens to identify the molecular nature of NSC1.

The high affinity NH_4^+ transport yeast mutants 31019b, 26972c and 26972c2, were tested for survival on media containing 100 mM MA^+ at different pH and Ca^{2+} concentrations. Conditions that facilitate the accumulation of the toxic NH_4^+ analogue, MA^+, will result in reduced growth. If NSC1 is a pathway for MA^+ flux into yeast, which is likely based upon MA^+ flux through plant viNSCCs and NH_4^+ flux through NSC1 (Davenport, 1998; Bihler, 2002), changes in pH and $[Ca^{2+}]$ should influence the fitness of cells harbouring functional NSC1 proteins. This investigation revealed a growth depression in the strain 31019b that is not present in 26972c or 26972c2 when grown on media with 0.2 mM Ca^{2+} and at pH 7.0 (Figure 2-1). This suggests that 31019b has a functional NSC1 protein(s) and they act to facilitate the accumulation of MA^+ in the cell. These data also indicate that 26972c and 26972c2 may not have a functional NSC1 as their growth on media containing MA^+ is not influenced by pH or Ca^{2+}. This combined with the absence of NSC1 – like currents in spheroplasts of this strain when patch clamped (Kaiser et al., 1998) makes for a convincing argument that 26972c and its derivative 26972c2 are putative NSC1 null mutants.

Utilising this phenotypic difference, a *Glycine max* cDNA library was transformed into 26972c2 and transformants screened for the induction of a phenotype observed in 31019b in an attempt to isolate a NSC1 encoding cDNA. Plates were replicated to include a loading control and two plates containing 100 mM MA^+ at pH 7.0, one with 0.2 mM Ca^{2+} and the other with 10 mM Ca^{2+}. Visible growth differences as a result of changes in $[Ca^{2+}]$ were noted after a specified time period, compared with empty vector transformed controls for the cDNAs considered for NSC1 candidates.

Sequence analysis of cDNAs causing Ca^{2+}-dependent growth repression of the yeast grown on 100 mM MA^+ revealed no obvious NSC1 candidates.

Four cDNAs selected encoded a *Glycine max* leghaemoglobin gene (Table 2-1). They showed a general loss of fitness when grown on media containing MA^+, independent of the Ca^{2+} concentration (Figure 2-3). Expression of a peroxidase precursor protein, GmIPER1, resulting in increased MA^+ toxicity at low Ca^{2+} at pH 7.0. These data suggest free radicals such as reactive oxygen species (ROS) may be involved in the perceived MA^+ toxicity phenotype. Both of these proteins are involved in cellular oxygen manipulation and in the generation of ROS. Leghaemoglobins primarily act to minimise free oxygen in legume nodules, allowing activity of the oxygen-labile bacteroid enzyme nitrogenase (Ott et al., 2005). Whilst playing important roles in the sequestration of O_2 within the nodule, evidence exists of leghaemoglobins promoting the generation of ROS (Becana et al., 2000). GmIPER1 is a protein integral to the production of peroxidases, which in turn play a role in the cleavage of peroxide groups liberating ROS (Yi, 1998). The generation of ROS in plant cells can elicit the activation of NSCCs which are influenced by Ca^{2+} present in the surrounding media (Demidchik et al., 2003). It is possible that yeast cells may be similarly influenced by ROS, activating a native NSCC and inducing the phenotypes observed.

Other proteins that generated a phenotype of interest in the screens were a proline rich repeat protein and a MYB transcription factor. Whereas helix-loop-helix transcription factors, the class to which MYBs belong, have been associated with low affinity cation flux (Kaiser et al., 1998), it is unlikely these proteins are acting as channels. It is likely these proteins are interacting with the yeast system to reduce fitness on MA^+

containing media through perturbation of native biochemistry or processes. It is almost certainly not due to these proteins acting as a viNSCC.

The use of this screen failed to reveal the identity of a channel like protein that was a good candidate for a viNSCC. The two most likely reasons for this are the lack of sensitivity in the screen due to its large scale and the lack of knowledge concerning the putative NSC1 mutation. The expected phenotype for this screen is anticipated to be subtle and serial dilutions of yeast cultures are best suited to detect such subtleties, as observed by Bihler et al. (2002). The use of large-scale array equipment revealed a great deal of equipment-sourced variation in the data collected (Figure 2-2 A and B). This was most apparent on the loading control plates (Figure 2-2 B). Each well was inoculated from a single colony growing on the same media as the loading control plates so each colony should grow well. When replica plated, many colonies grew poorly on the loading control. This was either a result of the replica plating process or an artefact of growing the yeast in 384 well plates with glycerol added to the media.

If the lack of NSC1 phenotype in 26972c or 26972c2 is a result of mutations outside of a direct mutation of the gene encoding NSC1 itself (i.e. yeast signalling, protein targeting, and or post translational modification processes) may make data interpretation difficult. A number of cDNAs that do not encode transport proteins were identified and describing their roles in the phenotype was clearly outside the aim of this experiment. Failure to find any strong candidates combined with difficulty with methodology resulted in this approach being abandoned.

Identification of the viNSCC in yeast was attempted using an alternate approach. A transposon insertion yeast cDNA library was sourced (Kumar et al., 2004) and this was used to randomly mutate the genome of the yeast strain 31019b through homologous recombination. Colonies that displayed improved growth on media containing MA^+ with low Ca^{2+} and at pH 7.0, showing a phenotype putatively associated with the disruption of NSC1 activity, were selected. All transformants selected were shown to have mutations in components of the 60s ribosomal RNA subunit of *S. cerevisiae*. As rRNAs perform a large range of roles within the cell, drawing a single conclusion with respect to the phenotypes observed is difficult. The transposon mutation library appropriated for this investigation either contains so many copies of rRNA sequence that almost all transformants contain these proteins or the 60S subunit play an enigmatic role in MA^+ sensitivity in yeast.

Reliance on the use of heterologous gene expression in the yeast system developed here failed to reveal a convincing viNSCC candidate protein. This may have been due to Ca^{2+} concentration and a relatively narrow pH range being used as a screen. As many biochemical processes in yeast are sensitive to Ca^{2+} concentrations and to a greater extent pH, any phenotypes identified using this screen could be due an effect that is not due to viNSCC activity. To enhance the probability of revealing the identity of these proteins, the screen was further developed to incorporate bioinformatic approaches to better define possible candidates prior to testing in yeast (Chapter 3).

2.4. Materials and Methods

2.4.1. Yeast Phenotyping on Solid Media. Comparison of MA$^+$ toxicity phenotype between 31019b, 26972c, 26972c2 and ∑1278b

Cells of 31019b, 26972c, 26972c2 and ∑1278b were grown to OD$_{600}$ of 2 in YPD and a series of 5 µL decimal dilutions applied to yeast minimal media agar plates minus Ca^{2+} supplemented with D-galactose (2 g/L). Plates contained 100 mM MA$^+$ and either 0.2 mM CaCl$_2$ or 10 mM CaCl$_2$ and were made with a range of media pH from 4.5 to 9.0. This was achieved using a Grensons solution 1 (Dubois, 1979) without adjusting pH and the subsequent addition of KOH to reach the pH required. Phenotypic differences were apparent between pH 6.5 and 7.0 only (Figure 2-1).

2.4.2. Phenotyping *Glycine max* cDNAs expressed in 26972c2

Saccharomyces cerevisiae strain 26972c2 was transformed with a *Glycine max* nodule cDNA library (Kaiser et al., 1998) using the high efficiency lithium acetate / polyethylene glycol method (Woods). A total of 2 x 10^6 transformants were plated on to media containing yeast nitrogen base (BD Biosciences) + 2% (w/v) agar + 2% (w/v) glucose. Colony density was calculated to be 4000 to 5000 colonies per 23 cm^2 plate. Colonies were grown at 28°C for 3 days. Clearly separated colonies were then picked using a VersArray Colony Picking Array System (BioRad) and grown in 384 well plates containing yeast nitrogen base (BD Biosciences) + 2% (w/v) glucose + 10% (v/v) glycerol at 28°C for 3 days. A total of 66 384 well plates were picked. Controls of 26972c2 and 31019b transformed with the empty pYES3 vector were also included. Arrays of 5µL drops from each well of the 384 well plates were replicated

on to 23 cm^2 plates containing minimal media (Grenson, 1966) + 100 mM MA$^+$, 0.1% (w/v) L-proline and 2% (w/v) D-galactose at pH 7.0 with either 0.2 mM or 10 mM Ca^{2+}. Yeast were also grown on solid media containing yeast nitrogen base (BD Biosciences) + 2% (w/v) glucose as a loading control. Plates were incubated for 5 days at 28°C and colonies photographed using the Chemidoc XRS imaging system (Biorad). Images of each replicate were overlayed using Imagene (BioDiscovery, USA) and differences in growth quantified. Data was filtered initially by subtracting as null results replicants that failed to grow on yeast nitrogen base + 2% glucose plates or grew poorly compared to the MA$^+$ containing plates. Of the remaining colonies, the relative colony size was compared between the 0.2 mM Ca^{2+} and the 10 mM Ca^{2+} replicons using ImaGene software (BioDiscovery, USA) and those that displayed a reduced growth phenotype under the 0.2 mM Ca^{2+} conditions were selected as possible candidates for expression of NSC1 (Figure 2-2).

Confirmation of putative viNSCC phenotype was done. Aliquots from the original 384 well plates, stored at -80°C, and grown to saturation in yeast nitrogen base (BD Biosciences) + 2% (w/v) D-glucose at 28°C and cells from 10 ml of the saturated solution were pelleted through centrifugation at 4000 x g for 4 minutes and the pellet washed twice with 10 ml of sterile MilliQ water. The pellet was then resuspended in sterile water and diluted to an OD$_{600}$ of 0.4. Decimal dilutions were made to a final concentration equivalent to OD$_{600}$ = 0.0004 and 10 μL samples plated on to yeast nitrogen base (BD Biosciences) + 2% (w/v) D-glucose and Grensons (Grenson, 1966) + 100 mM MA$^+$, 0.1% (w/v) L-proline and 2% (w/v) D-galactose at pH 7.0 with either 0.2 mM Ca^{2+} or 10 mM Ca^{2+} (Figure 2-3).

Plasmid DNA was extracted from positive colonies by vortexing in yeast breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) with the addition of approximately 50 µL of glass beads. An equal volume of phenol / chloroform / isoamyl alcohol (25:24:1 v/v/v) was added and the solution mixed well by inversion. The mixture was transferred to a 2 ml heavy phase lock tube (Eppendorf) and centrifuged at full speed for 30 seconds. An additional 200 µL of chloroform was then added, mixed by inversion and then re-centrifuged in a microfuge at full speed for 30 seconds. 1 µL of supernatant was then used to transform the *E. coli* strain DH5α via the heat shock method (Maniatis, 1989). Colonies selected by growth in the presence of ampicillin (100 mg/L) were then grown in liquid culture and their plasmid DNA extracted and re-transformed into 26972c2.

Re-transformed cells as well as pYES3-transformed 31019b were grown to saturation in yeast nitrogen base (BD Biosciences) + 2% (w/v) D-glucose, pelleted by centrifugation and washed in sterile milliQ water. Cells were resuspended in sterile milliQ water to an OD_{600} of 0.4 and decimal dilutions performed to a final density of $OD_{600} = 4 \times 10^{-5}$. A 5 µL sample of each was plated with the exception of the plates that yielded P10 A10.8 and P9 E11.5 which were performed using a 96 pin replicating head (Figure 2-3).

2.4.3. Phenotyping transposon mutations of 31019b for improved MA^+ tolerance.

Strain 31019b was transformed with a *Saccharomyces cerevisiae* transposon insertion cDNA library (Kumar et al., 2004) using the lithium acetate / poly ethylene glycol

method (Gietz, 1995) to a relative number of 10^6 transformants. Transformants were screened for improved growth when plated on solid minimal media (Grenson, 1966) with 0.1% L-proline, 100 mM MA$^+$, 2% D-glucose, 0.2 mM Ca^{2+} and at a pH of 7.0. Fourteen colonies were selected and grown overnight in yeast nitrogen base (BD Biosciences) + 2% (w/v) D-glucose. An empty vector control of 31019b was also grown. Cultures were pelleted and washed twice in sterile milliQ water and resuspended to OD$_{600}$ of 0.3. Decimal dilutions to a final OD$_{600}$ of 0.003 were made and 5 µL of each placed on solid yeast minimal media with 100 mM MA$^+$, 0.1% (w/v) L-proline, 0.2 mM Ca^{2+}, pH 7.0. The transposon insertion sites of six candidates were investigated further using inverse PCR. Each candidate was grown overnight in YPAD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.02% (w/v) adenine hemisulfate) and genomic DNA was extracted from each candidate. Genomic DNA was digested with *Hind* III or *EcoR1* (Promega) overnight at 37°C and the restriction enzyme deactivated by heating to 65°C for 20 minutes. DNA was then circularised using T4 ligase (Promega) overnight at 4 °C. PCR was performed on the ligated DNA with a temperature protocol of 95 °C 30 s, 55 °C 1.5 minutes, 72 °C 1 minute x 35 cycles and with the primers mTnLB945 (GTATCGCTCGCCACTTCAACAT) and mTnRB1268 (GCCATCGCCATCTGCTGC) using platinum Taq (Invitrogen). Amplified bands were gel extracted by Freeze and Squeeze (BioRad), ligated into pGEMT-Easy (Invitrogen) and transformed into *E. coli* strain DH5α. Positive clones were selected through survival on media containing ampicillin and through blue / white selection (Maniatis, 1989). Positive colonies were selected, grown up and plasmid DNA extracted using a commercial spin column kit (Sigma GenElute Plasmid Miniprep Kit). Inserts were confirmed through digestion with *Eco*R1 (Promega) at 37°C for 1

hour. M13 forward and reverse primers were used in conjunction with Big Dye v3.2

(Applied Biosystems) for sequencing.

Clone Name	Accession	Function
P2 B7. 1	GI:18595	Leghaemoglobin 1
P9 E11.5	GI:169994	Leghaemoglobin c-2
P10 A10.8	GI:170048	Proline Rich Protein
P2 A2.6	GI:2245682	Peroxidase Precursor Protein
P7 B8.22	GI:110931719	MYB Transcription Factor

Table 2-1

Table listing genes that, when over-expressed in the S. cerevisiae strain 26972c2, elicited a growth response similar to that predicted for cells over-expressing a viNSCC.

Clone Name	Accession	Function
mTn 1	RDN25-2	Component of the 60s ribosomal subunit
mTn 4	YMR418w (RPL12b)	Component of the 60s ribosomal subunit
mTn 5	YDR418w (RPL12b)	Component of the 60s ribosomal subunit
mTn 9	YDR418w (RPL12b)	Component of the 60s ribosomal subunit
mTn 11	YDR418w (RPL12b)	Component of the 60s ribosomal subunit

Table 2-2

Table showing the identities of genes that, when disrupted by transposon insertion, induce a phenotype in the yeast strain 31019b similar to that expected from the disruption of viNSCC activity.

C

	10 mM Ca²⁺			0.2 mM Ca²⁺		
pH	Neat	1/10	1/100	Neat	1/10	1/100
6.0						
6.5						
7.0						
8.0						
8.5						

D

	10 mM Ca²⁺			0.2 mM Ca²⁺		
pH	Neat	1/10	1/100	Neat	1/10	1/100
6.0						
6.5						
7.0						
8.0						
8.5						
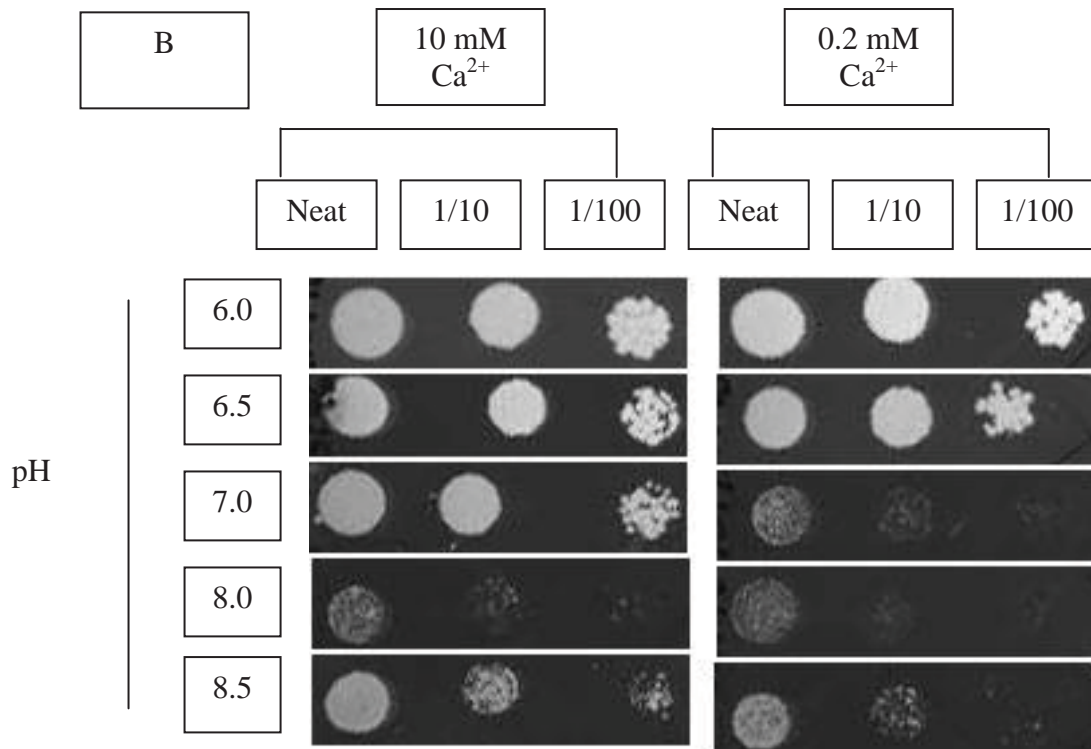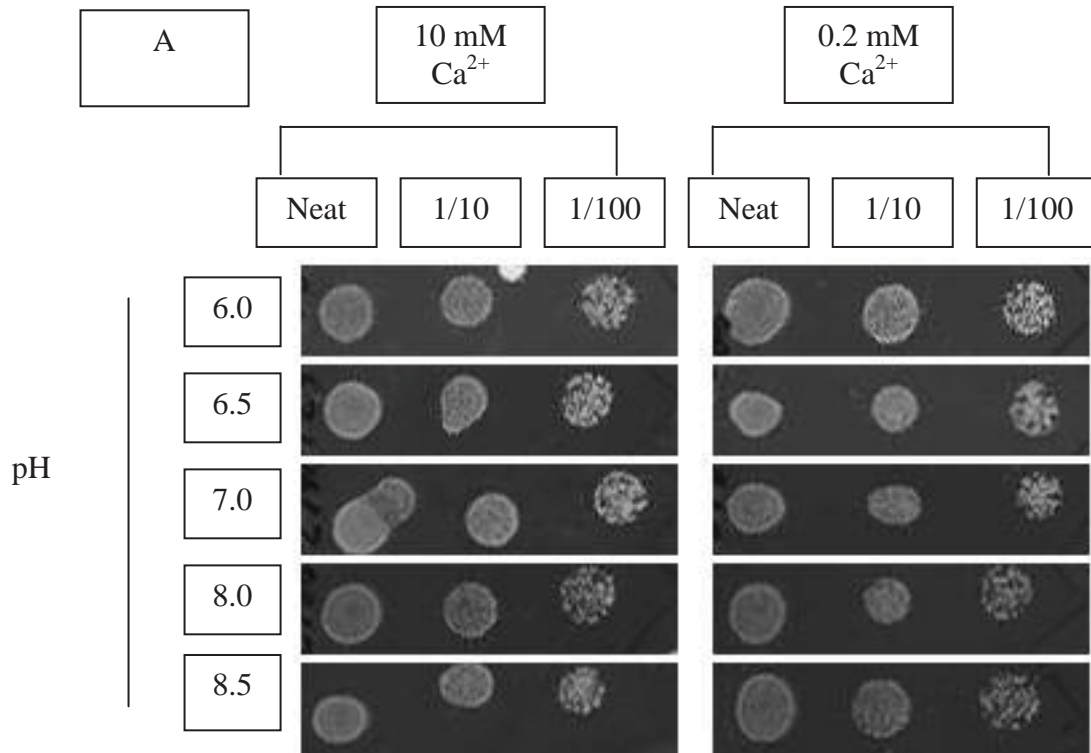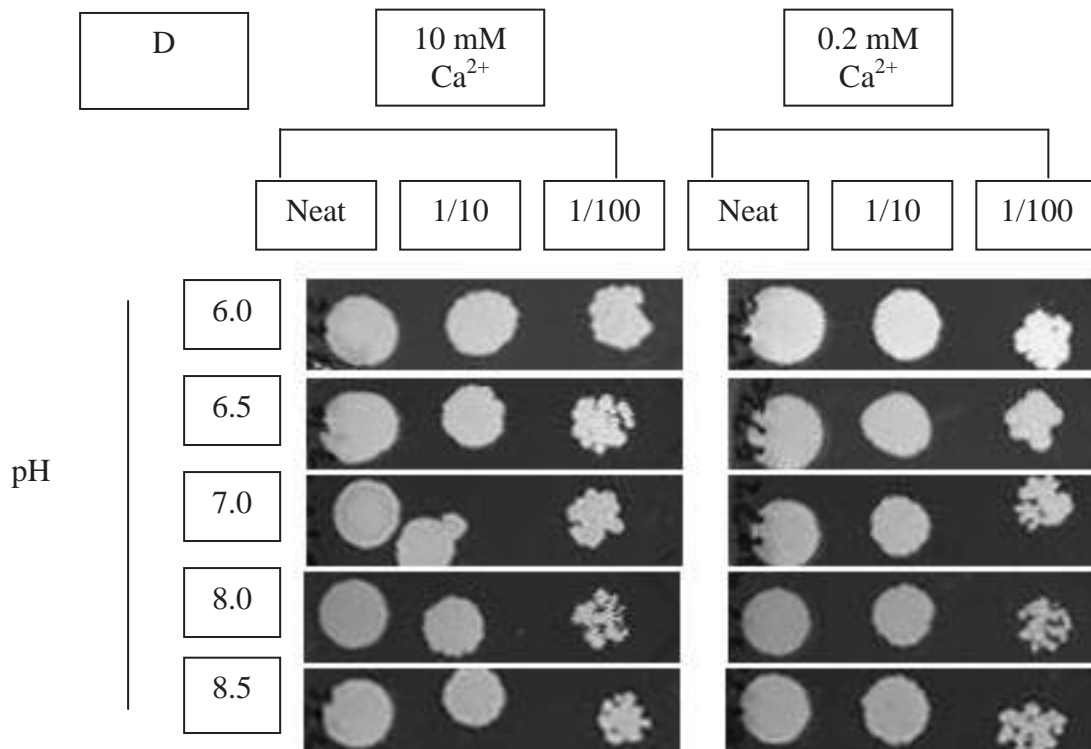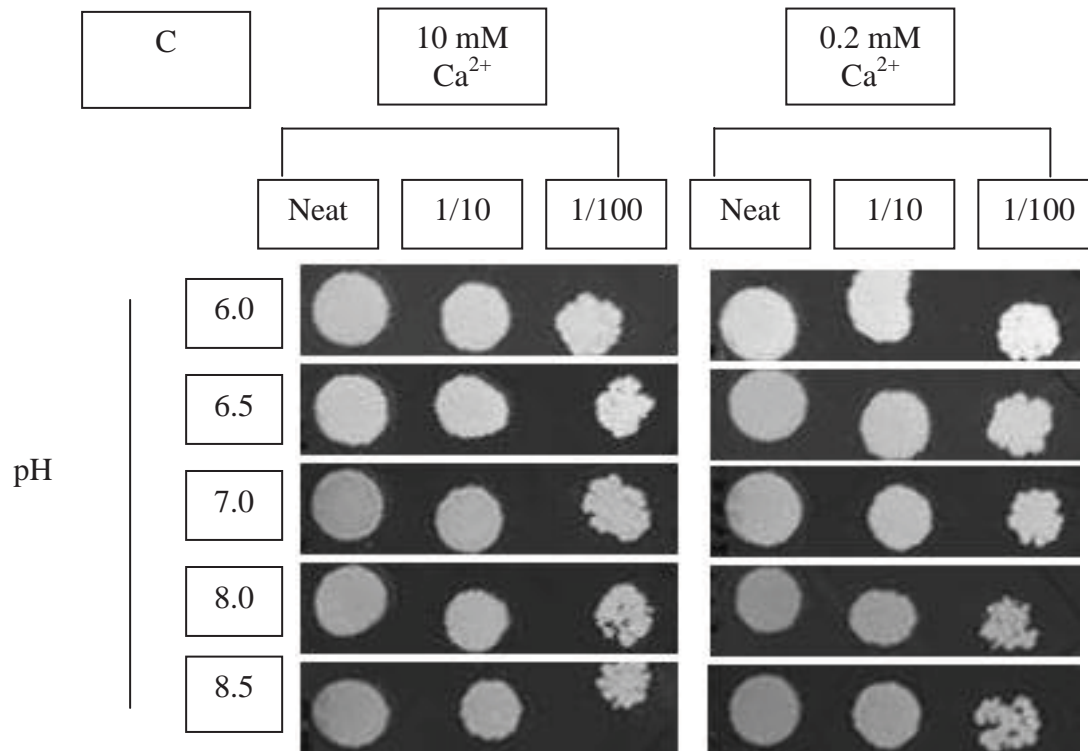
Figure 2-1: The influence of media pH and Ca^{2+} concentration on MA^+ toxicity phenotypes of different *S. cerevisiae* strains.

Strains $\sum$1278b (A), 31019b (B), 26972c2 (C) and 26972c (D) were grown, serially diluted and plated as described in (section 2.4.1). Restriction of colony growth was used as a measure of susceptibility to MA^+ toxicity. $\sum$1278b (A) grew very poorly, with only a 'watermark' of dead cells visible on the plates. The absence of contrasting growth causes the cell growth to appear more robust than it actually was. 31019b (B) showed increased susceptibility to MA^+ at pH 7.0 and higher under 0.2 mM Ca^{2+} conditions. This susceptibility was ameliorated in the presence of 10 mM Ca^{2+}, with MA^+ toxicity becoming pronounced at pH 8.0 and higher under these conditions. The strains 26972c2 and its parent line 26972c showed no changes in MA^+ toxicity as a function of altered pH of Ca^{2+}.
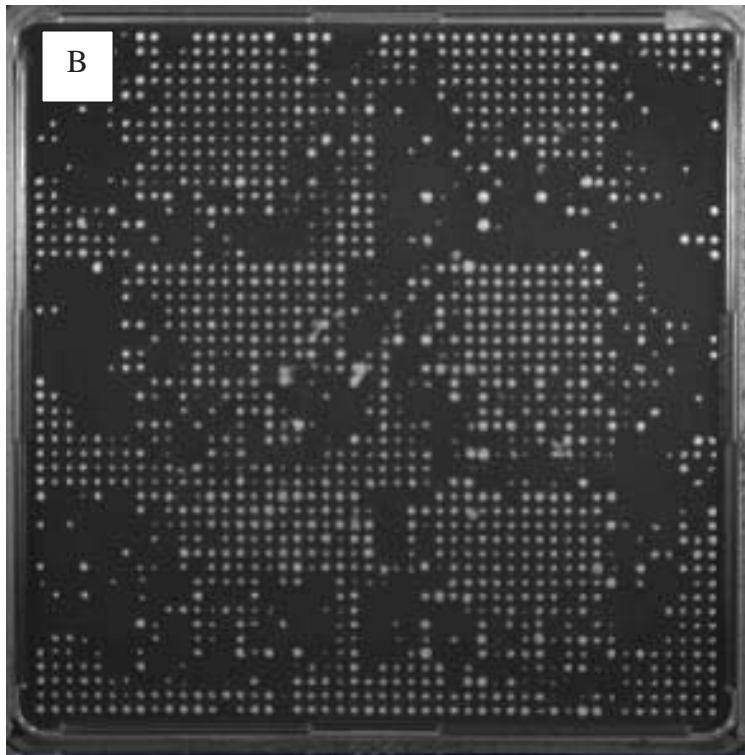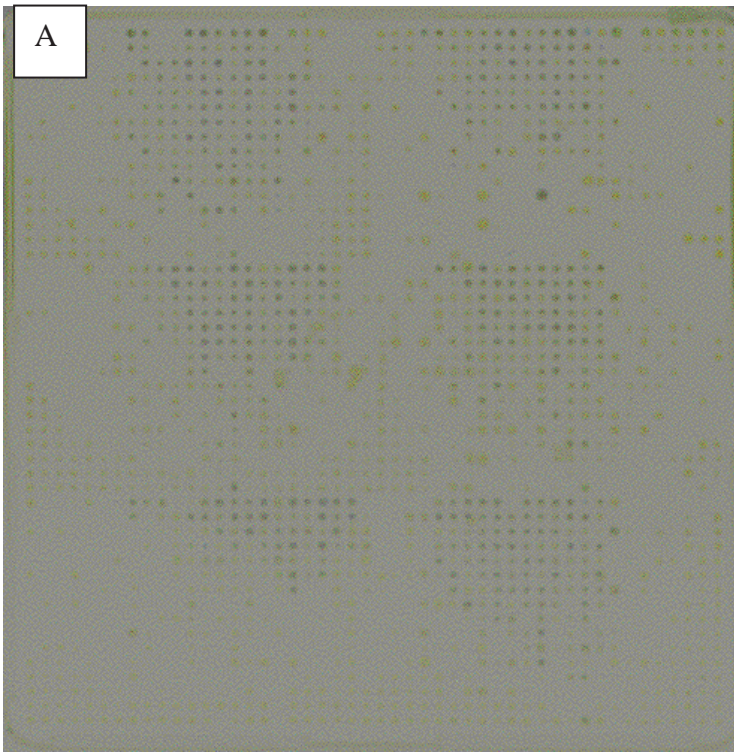
Figure 2-2: Representative plates of S. cerevisiae strain 26972c2 transformed with a *Glycine max* cDNA library and arrayed using the BioRad Colony Picking and Array System.

Yeast colonies were also replica plated onto media containing MA^+ with different Ca^{2+} concentrations (section 2.4.2). These images were overlayed using Imagene (BioDiscovery, USA) software that produces an overlayed colour image (A). Colonies that displayed increased MA^+ toxicity phenotypes under low Ca^{2+} conditions were selected and the cDNA in the host plasmid identified.

Loading control plates of YNB + 2% Glucose were created for each of the 11 arrays examined (B). Each plate has 6 x 384 well plates. Under these conditions, all transformants should grow with equal vigour as the presence of glucose strongly represses the Gal1 promoter in pYES3, the plasmid into which the cDNA library has been transformed. Variation in colony growth is indicative of erroneous replication by the arraying system used.
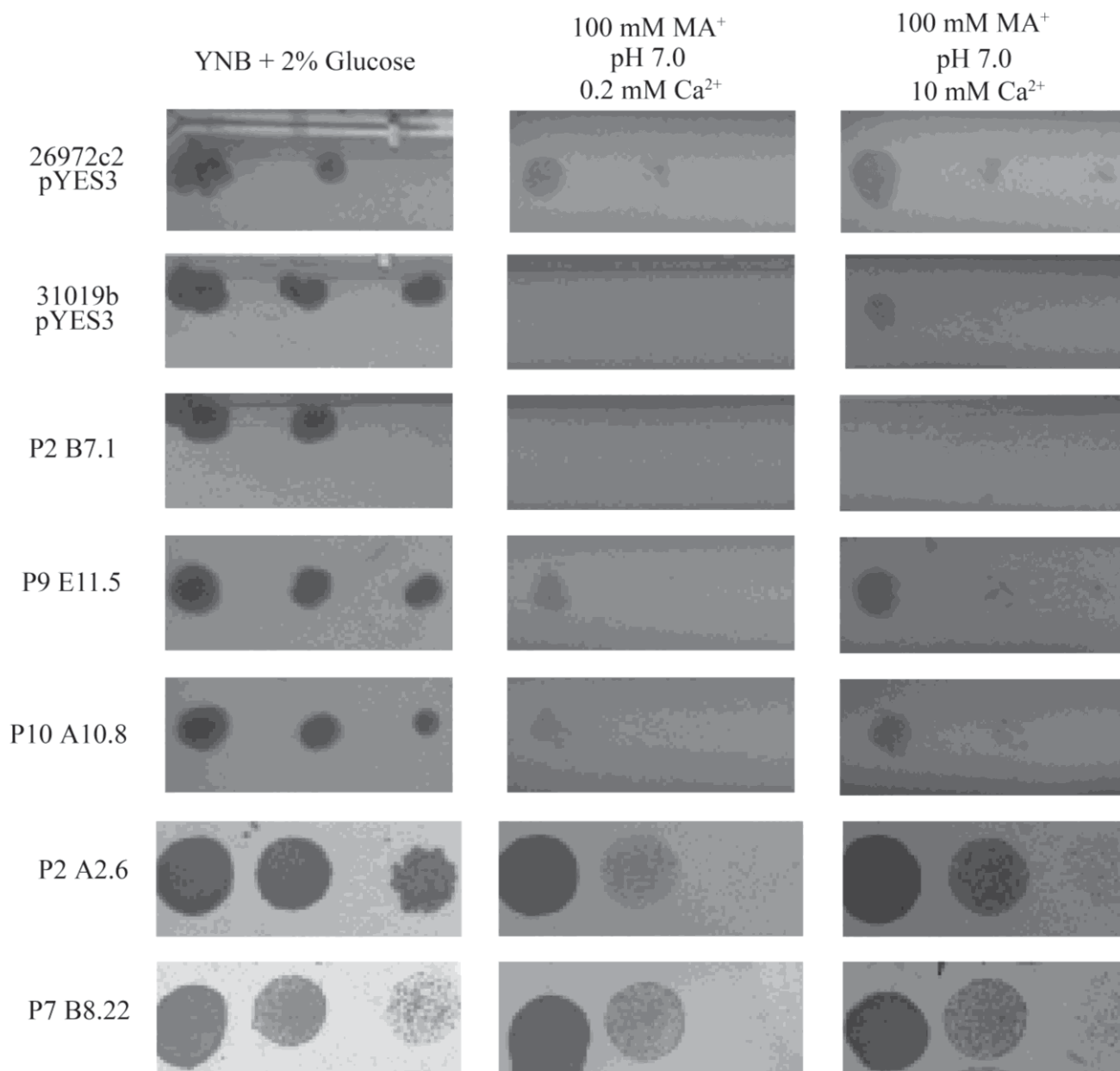
Figure 2-3: Confirmatory screening of increased MA$^+$ toxicity phenotypes under 0.2 mM Ca^{2+} conditions of *S. cerevisiae* strain 26972c2 transformed with a *Glycine max* cDNA library.

Transformed yeast showing increased MA$^+$ toxicity were plated via serial dilutions onto minimal media containing, 100 mM MA$^+$, 0.1% L-proline at a pH of 7.0 with either 0.2 mM Ca^{2+} or 10 mM Ca^{2+} (section 2.4.2). Expression of the cDNA in P2 B7.1 gave increased MA$^+$ toxicity for both concentrations of Ca^{2+} tested. P9 E11.5

and P10 A10.8 revealed a clear increase in MA^+ toxicity at 0.2 mM Ca^{2+} compared to 10 mM Ca2+ P2 A2.6 and P7 B8.22 showed a similar response but with a much reduced contrast between Ca^{2+} concentration responses. Differences in colony size apparent for P2 A2.6 and P7 B8.22 are the result of manual pipetting 5 μL of liquid culture per spot. The remainder of the colonies shown were produced through the use of a 96 pin replicator.

A) P2 B7.1

>emb|V00454.1|GMGL04 Glycine max leghemoglobin gene (gene I) from clone Ch4
GmLb11
Length=1204

```
 Score = 99.0 bits (53),  Expect = 4e-17
 Identities = 82/104 (78%), Gaps = 1/104 (0%)
 Strand=Plus/Plus


Query  209  ATACTGGGGAAAGCNACCTGNGGCANAGGACTTGTTCTCATTTCTATNAAATGGANTANA  268
            |||||||  ||||||  |||||   |||  |||||||||||||||||||||   |||||||  || |
Sbjct  411  ATACTGGAGAAAGC-ACCTGTAGCAAAGGACTTGTTCTCATTTCTAGCAAATGGAGTAGA  469


Query  269  CNGCNNTAANCNNNAGCTCGNGGGNCNTGCTGAAAAACNTTTTG  312
            |   |   ||| |    |||||   ||| |  |||||||||||  |||||
Sbjct  470  CCCCACTAATCCTAAGCTCACGGGCCATGCTGAAAAACTTTTTG  513
```

>emb|V00453.1|GMGL03 Glycine max leghemoglobin gene (gene Lba)
Length=1886

```
 Score = 93.5 bits (50),  Expect = 2e-15
 Identities = 81/104 (77%), Gaps = 1/104 (0%)
 Strand=Plus/Plus


Query  209  ATACTGGGGAAAGCNACCTGNGGCANAGGACTTGTTCTCATTTCTATNAAATGGANTANA  268
            |||||||  ||||||  |||||   |||  |||||||||||||||||||||   |||||||  || |
Sbjct  333  ATACTGGAGAAAGC-ACCTGCAGCAAAGGACTTGTTCTCATTTCTAGCAAATGGAGTAGA  391


Query  269  CNGCNNTAANCNNNAGCTCGNGGGNCNTGCTGAAAAACNTTTTG  312
            |   |   ||| |    |||||   ||| |  ||||||||||| |  |||||
Sbjct  392  CCCCACTAATCCTAAGCTCACGGGCCATGCTGAAAAGCTTTTTG  435
```

>gb|J01302.1|SOYLBC3 soybean leghemoglobin c-3 gene
Length=1118

```
 Score = 93.5 bits (50),  Expect = 2e-15
 Identities = 81/104 (77%), Gaps = 1/104 (0%)
 Strand=Plus/Plus


Query  209  ATACTGGGGAAAGCNACCTGNGGCANAGGACTTGTTCTCATTTCTATNAAATGGANTANA  268
            |||||||  ||||||  |||||   |||  ||||||||||||||||||||     ||||||  || |
Sbjct  323  ATACTGGAGAAAGC-ACCTGTAGCAAAGGACTTGTTCTCATTTCTAGCTAATGGAGTAGA  381


Query  269  CNGCNNTAANCNNNAGCTCGNGGGNCNTGCTGAAAAACNTTTTG  312
            |   |   ||| |    |||||   ||| |  |||||||||||  |||||
Sbjct  382  CCCCACTAATCCTAAGCTCACGGGCCATGCTGAAAAACTTTTTG  425
```

B) P9 E11.5

>gb|AF007211.1| Glycine max peroxidase precursor (GMIPER1) mRNA, complete
cds
Length=1287

 GENE ID: 547499 GMIPER1 | peroxidase, pathogen-induced [Glycine max]
(10 or fewer PubMed links)

```
 Score = 1363 bits (738),  Expect = 0.0
 Identities = 825/869 (94%), Gaps = 23/869 (2%)
 Strand=Plus/Plus
```

```
Query  60   GGCA-GAGAGAGAGAGAAATGAAGTCTTTCCATCTCACAGTGACAGCTTTGTGCTGTGTA  118
            ||||  |||||||||  |||||||||||||||||||||||||||||||||||||||||||
Sbjct  7    GGCACGAGAGAGAGAAAAATGAAGTCTTTCCATCTCACAGTGACAGCTTTGTGCTGTGTC  66

Query  119  GTGGTTGTGCTTGGAGTGCTTCCACTCTCCCTAGATGCACAACTAGATCCCTCCTTTTAC  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   GTGGTTGTGCTTGGAGTGCTTCCACTCTCCCTAGATGCACAACTAGATCCCTCCTTTTAC  126

Query  179  AGAGACACTTGTCCCAGGGTTCACTCCATTGTTCGTGAAGTCGTCAGGAACGTTTCAAAG  238
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  AGAGACACTTGTCCCAGGGTTCACTCCATTGTTCGTGAAGTCGTCAGGAACGTTTCAAAG  186

Query  239  AAAGATCCCCGTATGCTTGCTAGTCTCATTAGACTTCACTTTCATGACTGCTTTGTTCAA  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  AAAGATCCCCGTATGCTTGCTAGTCTCATTAGACTTCACTTTCATGACTGCTTTGTTCAA  246

Query  299  GGATGTGATGCATCAGTTTTGTTGAACAACACTGCCACCATAGAGAGCGAGCAACAAGCT  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  GGATGTGATGCATCAGTTTTGTTGAACAACACTGCCACCATAGAGAGCGAGCAACAAGCT  306

Query  359  CTGCCAAACAACAACTCACTAAGAGGTTTGGATGTTGTGAACGACATTAAGACAGCAGTG  418
            ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  307  CTGCCAAACAACAACTCACTAAGAGGTTTGGATGTTGTGAACTACATTAAGACAGCAGTG  366

Query  419  GAAAAGGCTTGTCCCGGCGTGGTTTCTTGTGCTGATATTCTTACCCTTGCATCCGAAATA  478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  367  GAAAAGGCTTGTCCCGGCGTGGTTTCTTGTGCTGATATTCTTACCCTTGCATCCCAAATA  426

Query  479  TCTTCTGTTCTGGGTGGTGGTCCTGATTGGAAAGTTCCATTGGGAAGAAGAGATAGTTTA  538
            |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  427  TCTTCTGTTCTGGGTGGTGGTCCTCATTGGAAAGTTCCATTGGGAAGAAGAGATAGTTTA  486

Query  539  ACAGCAAACCGAAACCTTGCAAATCAAAACCTTCCAGCTCCATTCTTCAACCTATCTCGA  598
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ACAGCAAACCGAAACCTTGCAAATCAAAACCTTCCAGCTCCATTCTTCAACCTATCTCGA  546

Query  599  CTTAAAGCTGCCTTTGCTGTTCAAGGTCTTGACACCACTGATCTAGTTGCACTCTCAGGT  658
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  CTTAAAGCTGCCTTTGCTGTTCAAGGTCTTGACACCACTGATCTAGTTGCACTCTCAGGT  606

Query  659  GCTCATACATTCGGCAGAGCTCACTGCAATTTCATTCTTTGGACCGATTGTTACAATTTC  718
            |||||||||||||||||||||||||||||||||||||| | ||||||||| |||||||||
Sbjct  607  GCTCATACATTCGGCAGAGCTCACTGCAATTTCATTCTT-G-ACCGATTGT-ACAATTTC  663

Query  719  AGTGGAACTGGCCAAACCTGATCCAACTCTTGA-ACAAACTTACTTACAACAATTGGCNC  777
            |||||||||||| ||||||||||||||||||| |||| |||||||||||||||| | |
Sbjct  664  AGTGGAACTGGC-AAACCTGATCCAACTCTTGACACAA-CTTACTTACAACAATTG-CGC  720

Query  778  CAAANATGCCCCAATGGTGGACCTAAACAACCTTGTCAAATTNGGACCCTGTCNCNCCCG  837
            |||| |||||||||||||||||||| |||||||||| || | ||||||||| | || |
Sbjct  721  CAAATATGCCCCAATGGTGGACCTAA-CAACCTTGTCAATTTTG-ACCCTGTCACTCCTG  778

Query  838  GATNAAAATN-ANAAGGGNCTACNTCNCCAATCTTNCAGGTTTAANAAGGGNCTTTNCTT  896
             || |||||  | | ||| |||| || || |||||||| |||||| || ||||| |||  |||
Sbjct  779  -AT-AAAATTGACA-GGGTCTACTTCTCCAATCTT-CAGGTT-AAGAAGGG-CTTG-CTT  831

Query  897  NAAGAGTGGATCNAANAGNCTGTTTCTCG   925
             | ||||| ||| || || ||||| ||||
Sbjct  832  CA-GAGTG-ATC-AAGAG-CTGTT-CTCG   855
```

C)   P10 A10.8

>gb|J01301.1|SOYLBC2 Soybean leghemoglobin c-2 gene
Length=1292

```
 Score = 65.8 bits (35),  Expect = 4e-07
 Identities = 39/42 (92%), Gaps = 0/42 (0%)
 Strand=Plus/Plus


Query  273  TCCTAAGCTCACGGGCCATGCTNAAAANCTTTTTGCATTGGT  314
            ||||||||||||||||||||| |||| ||||||| ||||||
Sbjct  462  TCCTAAGCTCACGGGCCATGCTGAAAAGCTTTTTGGATTGGT  503
```

D)   P2 A2.6

>gb|J02746.1|SOYPRP1 Glycine max SbPRP1 gene encoding a proline-rich
protein, complete
cds
Length=2835

```
 Score =  750 bits (406),  Expect = 0.0
 Identities = 412/415 (99%), Gaps = 2/415 (0%)
 Strand=Plus/Plus


Query  62    AAGCCACCAGNTGTACAAGCCACCAGTGTACAAACCACCAGTGTACAAACCACCGNTGTA  121
             ||||||||||  |||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  1768  AAGCCACCAG-TGTACAAGCCACCAGTGTACAAACCACCAGTGTACAAACCACCGGTGTA  1826

Query  122   CAAGCCCCCTGTTAAGAAACCTCCTATTTACAAACCACCTTACCCAAAGTACCCTCCAGG  181
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1827  CAAGCCCCCTGTTAAGAAACCTCCTATTTACAAACCACCTTACCCAAAGTACCCTCCAGG  1886

Query  182   CTCCAACTGAAGACACCCACCTGATTTTGAATTAAGTTAAGGGATGTATTAAGTATATGC  241
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1887  CTCCAACTGAAGACACCCACCTGATTTTGAATTAAGTTAAGGGATGTATTAAGTATATGC  1946

Query  242   TAAATAAGAGGTGTATCTACAACCATTGACTTTGTCATGCTCAAAAATTTAGTTTAATTT  301
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1947  TAAATAAGAGGTGTATCTACAACCATTGACTTTGTCATGCTCAAAAATTTAGTTTAATTT  2006

Query  302   ACAAATTATGGCAAGGGTTACACCTGGCTAGCTTCCGCGTTTAGATCATTTGTTTATAGT  361
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2007  ACAAATTATGGCAAGGGTTACACCTGGCTAGCTTCCGCGTTTAGATCATTTGTTTATAGT  2066

Query  362   GCTATGGCCACGACAAGAGTGTTATGGGTGCATGAATGTAAAACGAAAAAAAAAAAAAAT  421
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  2067  GCTATGGCCACGACAAGAGTGTTATGGGTGCATGAATGTAAAACGAAAAAAAAAAAAA-T  2125

Query  422   TGTGTTTATTTTGTACTTCAAGTTATGAATAAAAGAATTTCACTGTTTGTTTGTA  476
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2126  TGTGTTTATTTTGTACTTCAAGTTATGAATAAAAGAATTTCACTGTTTGTTTGTA  2180
```

E) P7 B8.22

 Score = 1426 bits (772), Expect = 0.0
 Identities = 966/1053 (91%), Gaps = 51/1053 (4%)
 Strand=Plus/Plus

```
Query  58    CTCTTTTTCTCTTAATTTCTCATCATATC-A-T-ATC-TAT-T--A-ATCT-T--ACACA  106
             ||||||||| |||||||||||| |||| | | ||| ||| |  | || | |  | |||
Sbjct  80    CTCTTTTTCTTTTAATTTCTCATCTTATCTATTAATCTTATATACATATATATAAAGACA  139

Query  107   TATATTA-AG--ACATAGATATAATTTTTTTCGTTTTATTTTTCCCTGCATGGGCTGTTA  163
             || || | ||    | ||||||||||| || |||||| || |||||| |||||||||| |
Sbjct  140   TAGATAATAGTCATATAGATATAA-TTATTTCGTTATA-TTTTCCTTGCATGGGCTG-T-  195

Query  164   AGATAAGATGTCTCGCACGTGCTCACTGTGCGGCAACAACGGCCACAACTCGCGGACATG  223
                |||||||||||||||||||||||| |||||||||||||||||||||| ||||||||
Sbjct  196   ---TAAGATGTCTCGCACGTGCTCACAGTGCGGCAACAACGGCCACAACTCACGGACATG  252

Query  224   CACCGACGGTGGCGCCGCCGCTTCCTGCGGCGGGTCTCCCAGGGAGAACGGCATCATGCT  283
             ||||||||||   |   | ||    || ||  |||||||| |||||||||||||||||||
Sbjct  253   CACCGACGGT---G--G-CG---CC-GC--CGGGTCTCCGAGGGAGAACGGCATCATGCT  300

Query  284   GTTCGGCGTAAGAGTTATGACCGAAGCTAACTCCTCCTTCCGGAAGAGCGCCAGCATGAA  343
             |||||||||||||||| ||||| |||||||||||||||||||| ||||||||||||||||
Sbjct  301   GTTCGGCGTAAGAGTCATGACGGAAGCTAACTCCTCCTTCAGGAAGAGCGCCAGCATGAA  360

Query  344   CAACCTCTCACAGTACGACGCGGAATCCAACGCCGCCGACGCCGGCTACGCCTCCGACGA  403
             ||||||||||||||||||||||||| | |||||||||||||||||||||||||||||||
Sbjct  361   CAACCTCTCACAGTACGACGCGGAGTTCAACGCCGCCGACGCCGGCTACGCCTCCGACGA  420

Query  404   CGTTGTTCATGCCTCCGGCCGCACGCGCGAGCGCAAGCGAGGTGTTCCTTGGACGGAGGA  463
             ||| ||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CGTCGTTCACGCCTCCGGCCGCACGCGCGAGCGCAAGCGAGGTGTTCCTTGGACGGAGGA  480

Query  464   AGAGCATAGGTTGTTCCTGTTAGGGTTACACAAAGTCGGAAAAGGAGATTGGAGAGGAAT  523
             ||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   AGAGCATAGATTGTTCCTGTTAGGGTTACACAAAGTCGGAAAAGGAGATTGGAGAGGAAT  540

Query  524   TTCTCGAAACTTCGTGAAAACTCGCACGCCTACTCAGGTCGCTAGTCACGCTCAGAAGTA  583
             |||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   TTCTAGAAACTTCGTGAAAACTCGCACGCCTACTCAGGTCGCTAGTCACGCTCAGAAGTA  600

Query  584   CTTCCTCCGCCGCCACAACCAGAACC-GCCGTCGCCGGAGATCTAGCCTCTTCGACATCA  642
             ||||||||||||||||||||||||| | || | || |||||||||| ||||||||||||
Sbjct  601   CTTCCTCCGCCGCCACAACCAGAATCTGCTGCCGGCGGAGATCTA-CCTCTTCGACATCA  659

Query  643   CCACGGGATACGGTGATGGAATCTTCTACTATAATGGAAGAAGAACAAGTTCCGCAAGAA  702
             |||| | ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   CCACCG-ATACGGTGATGGAATCTTCTACTATAATGGAAGAAGAACAAGTTCCGCAAGAA  718

Query  703   ACGATGGCGGCGCCTATCCCCGCCGCATATCCGCCTTTTCCCGGCGCGGGTTTTCCGATG  762
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   ACGATGGCGGCGCCTATCCCCGCCGCATATCCGCCTTTTCCCGGCGCGGGTTTTCCGATG  778
```

```
Query  763   AGTCTATCTCCGGTGGTGTTGCCGGTGAGTGGTGAGAGACTGACCAAGCCGATCAGGCCG  822
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   AGTCTATCTCCGGTGGTGTTGCCGGTGAGTGGTGAGAGACTGACCAAGCCGATCAGGCCG  838

Query  823   ACGCCGATTGTACCGGTGCCTCCGTCTTCGAAGATGGCTAATTTGAACTTGAAAGGGAA-  881
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   ACGCCGATTGTACCGGTGCCTCCGTCTTCGAAGATGGCTAATTTGAACTTGAAAGGGAAA  898

Query  882   GCTTCTCNGACAA-TCTCATTGAGC-TTTGCCGTTGTCGTTGAATCTGC-ATCGCCAACG  938
             |||||||| ||||| |||||||||| ||||||||||||||||||||||| ||||||||||
Sbjct  899   GCTTCTCGGACAAATCTCATTGAGCCTTTGCCGTTGTCGTTGAATCTGCCATCGCCAACG  958

Query  939   CCGCGGTCTCAGGATCAGTCGCCGGCNAN-AGTGGCCACTCGTCGCCGTCNTCGTCTTCG  997
             ||||||||||||||||||||||||| |  ||||||||||||||||||||| ||||||||||
Sbjct  959   CCGCGGTCTCAGGATCAGTCGCCGGCGAATAGTGGCCACTCGTCGCCGTCATCGTCTTCG  1018

Query  998   GGGGGGG-TTTTCAGN-TA-GTCTGCA-CGAANT-CGGCG-CGGGGGGGA-AGTATCATC  1050
             | || || ||||||||  || |||||||| |||| | ||||| |||||||||| ||||||||||
Sbjct  1019  GCGGCGGCTTTTCAGGCTATGTCTGCAGCGAAGTTCGGCGGCGGGGGGGGATAGTATCATC  1078

Query  1051  ACCGTTGCTTGAAA-GAAGGAA-CAAA-AACAA   1080
             | |||||||||||| ||||||| |||| |||||
Sbjct  1079  AGCGTTGCTTGAAAAGAAGGAAACAAAGAACAA   1111
```

Figure 2-4: BLAST alignments of *Glycine max* cDNAs shown to impart MA$^+$ toxicity phenotypes when over-expressed in the *S. cerevisiae* strain 26972c2.

cDNAs that resulted in an increased MA$^+$ toxicity phenotype (Figure 2-3) were extracted and sequenced (section 2.4.2). Alignments shown are taken from BLAST results retrieved from the NCBI Soybean database retrieved June 2008.
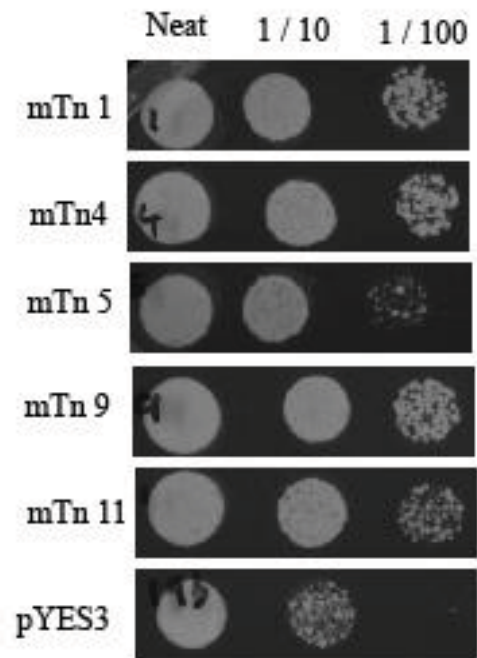
Figure 2-5: Improved MA$^+$ resistance in *S. cerevisiae* strain 31019b mutated with an mTn transposon library

S. cerevisiae strain 31019b was mutated with a yeast mTn transposon library (section 2.4.3) and transformants displaying improved resistance to MA$^+$ at pH 7.0 and in the presence of 0.2 mM Ca^{2+} selected (section 2.4.3). All mutants shown above showed similar degrees of resistance to MA$^+$ compared to the pYES3 empty vector control.

mTn1 – RDN25-2 25s Ribosomal RNA

```
mTn1:      301    GTTCCTCTGGGCGTCN-GCCCCCTTT-TCNGTTCGCCCTGGCCTGNNCGCTNTTCCTTCC 244
                  ||||   || || |   ||   |  || || || || |||||| ||    || ||||||||
RDN25-2: 462454   GTTCACCTTGGAGACCTGCTGCGGTTATCAGTACGACCTGGCATGAAAACTATTCCTTCC 462513


mTn1:      243    TGTGGNTTTTCNCGGGCCGTCNCNNGCGCNCCGGCGCCTGCNGNGGTGCTGGCCTCTTCC 184
                  ||||| ||||| |||||||||| |  |||| |||| ||| ||  |||||||||||||||||
RDN25-2: 462514   TGTGGATTTTCACGGGCCGTCACAAGCGCACCGGAGCCAGCAAAGGTGCTGGCCTCTTCC 462573


mTn1:      183    CGCCTTGNGNCCCCNTCTCCGGNTNNNCCNCTTCCGCGGGTGCTNNGCTGTT 132
                  ||| |   |  |||| ||||||| |   ||  |||||  ||||| |  ||||||
RDN25-2: 462574   AGCCATAAGACCCCATCTCCGGATAAACCAATTCCG-GGGTGATAAGCTGTT 462624
```


mTn4 – YDR418w (RPL12b) - Component of the 60s ribosomal subunit

```
mTn4:      217    GGAATTCAGAGGTATCAAAGTTACGTGNCCAATTGAAAATCCGAAACAGACGAAGCTGCT 276
                  ||||||||  ||||||||||||||||| || ||||||||||||| ||||||||| ||||||||
YDR418w: 1301761  GGAATTCAAAGGTATCAAAGTTAC-TGTCCAATTGAAAATCCAAAACAGAC-AAGCTGCT 1301818


mTn4:      277    GNTTCTGTTGTTCCATCTGCTTCCACTGTGGNCATNACTGCNTNGAAGGAACCACCAAAA 336
                  | ||||||||||||||||||||||| ||  ||| ||| ||||| | |||||||||||||||| |
YDR418w: 1301819  GCTTCTGTTGTTCCATCTGCTTCCTCTTTGGTCATTACTGCTTTGAAGGAACCACCAAGA 1301878


mTn4:      337    GACAGAAAGAGGG 349
                  |||||||||| ||
YDR418w: 1301879  GACAGAAAGAAGG 1301891
```


mTn5 – YDR418w (RPL12b) - Component of the 60s ribosomal subunit

```
mTn5:      159    GGAATTCANAGGTATCAAAGTTACTGTCCAATTGGAAAAATCCCGAAAACNAGA 212
                  ||||||||  |||||||||||||||||||||||| |||| |||  ||||| |||
YDR418w: 1301761  GGAATTCAAAGGTATCAAAGTTACTGTCCAATTG-AAAA-TCC--AAAAC-AGA 1301809
```


mTn9 – YDR418w (RPL12b) - Component of the 60s ribosomal subunit

```
mTn9:      184    GGAATTCCAAGGTATCAAAGTTACTGTCCAATTGAAAATCCAAAANAGACNAGCTGCTGC 243
                  |||||||  |||||||||||||||||||||||||||||||||||| |||| |||||||||
YDR418w: 1301761  GGAATTCAAAGGTATCAAAGTTACTGTCCAATTGAAAATCCAAAACAGACAAGCTGCTGC 1301820


mTn9:      244    TTCTGTTGTTCCATCTGCTTCCTCTTTGGNCATTACTGCTTTGGAAGGAACCACCNANAN 303
                  ||||||||||||||||||||||||||||| |||||||||||| |||||||||||| | |
YDR418w: 1301821  TTCTGTTGTTCCATCTGCTTCCTCTTTGGTCATTACTGCTTTG-AAGGAACCACCAAGAG 1301879


mTn9:      304    ACATAAAGAGGGNT 317
                  ||| ||||| || |
YDR418w: 1301880  ACAGAAAGAAGGAT 1301893
```

mTn11 – YDR418w (RPL12b) - Component of the 60s ribosomal subunit

```
mTn11:     172      GGAATTCAAAGGTATCAAAGTTACTGTCCAATTGAAAATCCAAAACAGACAAGCTGCTGC 231
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YDR418w: 1301761 GGAATTCAAAGGTATCAAAGTTACTGTCCAATTGAAAATCCAAAACAGACAAGCTGCTGC 1301820


mTn11:     232      TTCTGTTGTTCCATCTGCTTCCTCTTTGGTCATTACTGCTTTGAAGGAACCACCAAGAGA 291
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
YDR418w: 1301821 TTCTGTTGTTCCATCTGCTTCCTCTTTGGTCATTACTGCTTTGAAGGAACCACCAAGAGA 1301880


mTn11:     292      CAGAAAGAGGGGTCTGA-CGCTCA-GTGGAACGAAAAC-TCACGTTA-AGGCGGCCATTG 347
                    ||||||||| || |   || ||  ||| |    | || ||| || ||  ||    ||||
YDR418w: 1301881 CAGAAAGAAGGATAAGAACG-TCAAGCATAGCGGTAACATCCAATTGGATGAAATTATTG 1301939


mTn11:     348      AAGGTAGAAGAGAAA 362
                    ||  |   ||| |||
YDR418w: 1301940 AAATTGCCAGACAAA 1301954
```

Figure 2-6: BLAST alignments of *S. cerevisiae* genes shown to improved MA$^+$ tolerance in 31019b through their disruption by mTn insertion events.

Yeast were selected based upon their ability to survive better than the empty vector control on media with Grensons minimal media, 100 mM MA$^+$, 0.1 % L-proline, 0.2 mM Ca^{2+} at pH 7.0 (section 2.4.3). The site of transposon mutation was determined through iPCR (section 2.4.3), where amplified regions were cloned into the pGEMT-easy vector and sequenced. Alignments shown are taken from BLAST alignments of sequenced products against the *S. cerevisiae* genome.

3. An in silico screen for voltage insensitive non-selective cation channels (NSCCs)

3.1.   Introduction

The vast accumulation of readily accessible online biological data developed through the many 'omics' related fields has increased the rate of scientific discovery many fold and has catalysed functional genomics studies to be conducted across all organisms. Interlinking information about genomes, transcripts, phenotypes and many other parameters have resulted in a very powerful tool for novel gene discovery.

The viNSCC (<u>v</u>oltage <u>i</u>nsensitive <u>n</u>on <u>s</u>elective <u>c</u>ation <u>c</u>hannel) has been observed in many biological systems including *Saccharomyces cerevisiae*, wheat, soybean and *Arabidopsis* (Tyerman et al., 1995; Bihler, 1998, 2002; Demidchik, 2002a; Demidchik and Tester, 2002c; Essah et al., 2003{Siemen, 1993 #234). Unfortunately, genetic characterisation of this transport process in plants and yeast has been absent till recently.

Many conventional functional screens using heterologous expression systems rely upon strong growth related phenotypes working in defined transport backgrounds. The inherent passive flux catalysed by viNSCCs is a likely explanation for the failure of such screens to identify carrier(s) responsible for this flux. viNSCCs will probably impart only a slight phenotype and a complementation screen, common with the use of heterologous expression systems, is most likely not sensitive enough to resolve the functions of this class of protein.

Initial attempts to use a yeast heterologous screen to identify putative viNSCC transporters were unsuccessful (Chapter 2). A second indirect approach was then chosen that employed an *in silico* screen of publicly available genome databases. This screen was designed to mine both the *S. cerevisiae* and *A. thaliana* genome databases to highlight possible candidates of viNSCC within the two organisms.

Alternative screens have become available with the ascension of integrated genome databases. An excellent example of the use of such databases in the discovery of novel protein functions was the screen employed by Marini et. al., (2000) which identified homologous regions in the protein sequence between the plant high affinity NH_4^+ transporters, AMTs, and the human Rhesus associated proteins such as RhAG.

Data on viNSCCs is predominately from electrophysiological studies (Baud and Kado, 1984; Siemen, 1993; Bihler, 1998, 2002; Demidchik, 2002a; Demidchik and Tester, 2002c). Current traces due to the action of viNSCCs have enabled a number of broad conclusions to be drawn. They are proteins integrated in the plasma membrane. They show a great deal of non-selectivity towards mono-valent cations but are blocked by some divalent cations such as Ca^{2+}. They also show sensitivity to external pH, being blocked at low pH and active at pH 7.5 and above (Bihler, 1998). These physiological characteristics appear comparable across species, including between plant viNSCCs (Demidchik, 2002a) and yeast viNSCCs (Bihler, 1998). An *in silico* screen for viNSCCs was designed using these properties as guides.

3.2. Results

3.2.1. Screen of the *Saccharomyces cerevisiae* Genome Database (SGD)

Only 1123 of the approximately 6000 predicted ORFs are uncharacterised in the SGD as of February 2008 (www.yeastgenome.org). Using protein prediction software and localisation data available as part of the SGD, 57 of these unannotated ORFs are suspected to be associated with cellular membranes. It is likely that the *S. cerevisiae* viNSCC is amongst these 57 candidates as it is an uncharacterised protein, known to be functional in yeast and is incorporated into the intracellular membrane(s). Predictions are supplied by the InterPro database (www.ebi.ac.uk/**interpro**) and data partially verified through large scale GFP localisation experiments (Huh, 2003).

3.2.2. Comparative screen of the *A. thaliana* database

Proteins selected by screening the *S. cerevisiae* database were then compared to *A. thaliana* proteins and candidates chosen primarily due to high degrees of sequence and structural similarity. The yeast proteins of unknown function, which showed similarity to uncharacterised homologs in Arabidopsis (7 in total), were then selected as final candidates (Table 1). Of the 7 candidates, 6 genes were successfully isolated from a *S. cerevisiae* ORF cDNA collection (Gelperin, 2005), while the other ORF (YDR090c) was unable to be retrieved from glycerol stocks. The six candidate cDNAs were then analysed for viNSCC phenotypes (Chapter 4).

3.3.   Discussion

3.3.1.   Interrogation of online databases

The advent of integral genomics databases has increased the availability of extended datasets to the average researcher. The accumulated knowledge available in the *Saccharomyces* Genome Database (SGD – www.yeastgenome.org) and The Arabidopsis Information Resource (TAIR – www.arabidopsis.org) was used to perform an *in silico* screen of predicted proteins that have properties associated with the viNSCCs.

The SGD has a wide range of available search parameters. These often contain the results of various sequence analysis and prediction algorithms used to putatively assign function to unknown sequences. Within the database is a prediction of yeast ORFs encoding membrane-associated proteins. As the yeast genome has approximately 6000 predicted ORFs, selecting only the proteins of unknown function that are predicted to be membrane bound significantly reduced the number of candidates.

The *in silico* analysis identified 6 putative yeast ORFs which were conserved in both yeast and Arabidopsis (Table 3-1).

3.3.2. Preliminary analysis of viNSCC candidates

YDR090c, YDR352w and YOL092w

Sequence analysis of the candidate yeast genes and corresponding homologs in Arabidopsis identified multiple members of the PQ loop repeat family (Yeast: YDR090c, YDR352w and YOL092w; Arabidopsis: At4g20100 (NP_193743.1), At2g41050 (NP_850340.1 and At4g36850 (NP_568009.2)). Subsequent BLAST searches of the yeast genome revealed another similar PQ loop protein, YBR147w, which is very similar to YOL092w. PQ loop repeat proteins are predicted to be membrane bound with 6 or 7 trans-membrane domains, depending on trans-membrane prediction software and as suggested in hydropathy plots of the proteins (Figure 3-1). Transcript data available on the SGD shows responsiveness to the nutritional status of yeast for each of these genes (Table 3-1), including transcriptional variation as a response to changes in cell N status.

The Arabidopsis PQ loop repeat proteins selected share 24 – 30% similarity the yeast proteins investigated. Loci with data available in AtGenExpress (http://www.weigelworld.org/resources/microarray/AtGenExpress), At2g41050 and At4g20100, indicate expression throughout all plant tissue with particularly high levels in pollen and meristematic regions. Expression of these genes in response to abiotic stress and plant nutritional status showed little difference to control samples. One of these genes, At4g36850, is reported to be down regulated in response to fluctuations in Na^+, K^+ and Ca^{2+} in microarray experiments (Maathuis, 2003).

YOR291w

YOR291w was selected due to its high similarity with an unannotated Arabidopsis protein, NP_197752.1 (At5G23630). These proteins are similar to known cation transport ATPases in yeast (Spf1p) and Arabidopsis (At4G37640). YOR291w is also transcriptionally up regulated under N depletion in yeast (Gasch et al., 2000). As this protein seems likely to transport cations and possibly interacts with protons, it was a good candidate for the viNSCC. Recent annotations made to the NCBI genome database (http://www.ncbi.nlm.nih.gov/), however, suggest this protein is an ATPase.

YPL264c

YPL264c is very similar to the Arabidopsis protein designated AAm63171.1 (GI:21404671) in TAIR (P value = 9.1 x 10^{-12}). These proteins have a DUF6 / RhaT motif, which is associated with characterised drug / metabolite transporters (Marchler-Bauer and Bryant, 2004). When analysed (BLAST) against the NCBI genome database many similar proteins are shown to share sequence similarities but these are also uncharacterised. YPL264c is also transcriptionally down regulated in response to N depletion in yeast (Gasch et al., 2000). Therefore, YPL264c is probably a membrane bound transporter (Figure 3-1) and is good viNSCC candidate.

YGR149w

YGR149w shows a high degree of similarity to the Arabidopsis NP_198396.1 (At5G35460) (P value = 2 x 10^{-20}). Both the yeast and Arabidopsis proteins show no evidence of conserved domains or similarity to proteins of known function. viNSCC candidature is based solely on the protein being uncharacterised and predicted to be membrane bound (Figure 3-1).

YFL034w

YFL034w is an uncharacterised protein predicted to be membrane bound in the yeast cell (Sonnhammer, 1998). It is very similar to the uncharacterised Arabidopsis NP_849505 (At4G36210) with a P value of 3×10^{-45}. It contains a DUF 726 (domain of unknown function #726) domain and has been shown to interact with Rpp0p, a component of the ribosomal stalk (Aruna, 2004). Potential MA^+ toxicity phenotypes in yeast have been associated with ribosomal proteins (Chapter 2). Likely to be membrane bound (Figure 3-1), uncharacterised in both yeast and Arabidopsis and down regulated under N depletion conditions in yeast (Gasch et al., 2000), YFL034w displays many of the qualities as a potential viNSCC.

The screen developed previously (Chapter 2) was adapted into an assay for potential viNSCC activity in these genes. ORFs of interest were over-expressed in the S. cerevisiae strain 31019b and assayed for increased MA^+ associated toxicity, a phenotype potentially associated with increased viNSCC activity (Chapter 4).

3.4. Materials and Methods

3.4.1. In silico screen of the <u>S</u>accharomyces <u>G</u>enome <u>D</u>atabase (SGD) and <u>T</u>he <u>A</u>rabidopsis <u>G</u>enome <u>R</u>esource (TIGR) for potential viNSCCs.

Using existing search functions found on the SGD website, the yeast genome database was examined for confirmed or predicted open reading frames (ORFs). Uncharacterised ORFs were then selected and their predicted localisation examined using data generated from the InterPro database (www.ebi.ac.uk/**interpro**), available through the SGD. Those proteins most likely to be membrane bound were selected as being candidates for the viNSCC.

Each identified protein sequence was interrogated by BLAST analysis against TIGR databases. The WU-BLASTp algorithm (Gish, 1996 - 2004) was used to determine similarity between the protein sequences of candidates identified in yeast to proteins catalogued in Arabidopsis genome database. Matches were analysed for sequence similarity where those with a p value of less than 0.01 were selected as having some similarity with their yeast counterparts. Arabidopsis genes of known function were eliminated. These candidates were then functionally assayed in a *S. cerevisiae* based screen Chapter 4).

S. cerevisiae ORF	A. thaliana accession	Similarity at protein level (P value)	Potential Protein Class	Notes:
YDR090c	NP_850340	0.0006	PQ-loop repeat protein	• Down regulated in N depletion studies in yeast • Down regulated in yeast at stationary phase
YDR352w	NP_193743.1	2×10^{-15}	PQ-loop repeat protein	• Down regulated in N depletion studies in yeast • Down regulated in yeast at stationary phase
YOL092w	NP_850340	1×10^{-15}	PQ-loop repeat protein	• Up regulated in yeast N depletion studies • Up regulated in yeast during stationary phase
YOR291w	NP_197752.1	3×10^{-35}	Similar to P-type ATPase Spf1p	• Up regulated in yeast under N depletion • Interacts with opi3 and other secretory pathway proteins
YPL264c	AAm63171.1	9.1×10^{-12}	Similar to drug / metabolite transporter VAS14_11394 in *Vibrio angstum* S14	• Down regulated with N depletion in yeast
YFL034w	NP_849505.2	3×10^{-45}	Similar to many unknown proteins across phyla	• Known to interact with Rpp0p, a component of the ribosomal stalk • Down regulated with N depletion.
YGR149w	NP_198396.1	2×10^{-20}	Similar to many unknown proteins across phyla	• Down regulated in stationary phase

Table 3-1: Candidate genes selected using *in silico* screens of online databases

YDR352w

Kyte/Doolittle hydrophilicity: Window = 19

YOL092w

Kyte/Doolittle hydrophilicity: Window = 19

YBR147w

Kyte/Doolittle hydrophilicity: Window = 19

YDR090c

Kyte/Doolittle hydrophilicity: Window = 19
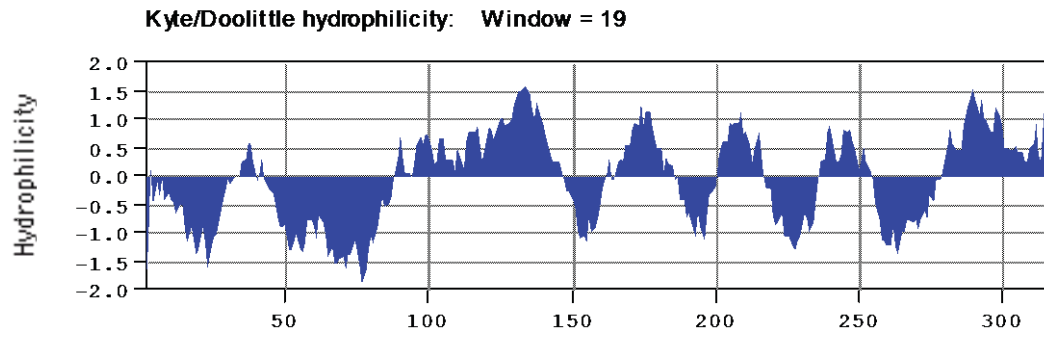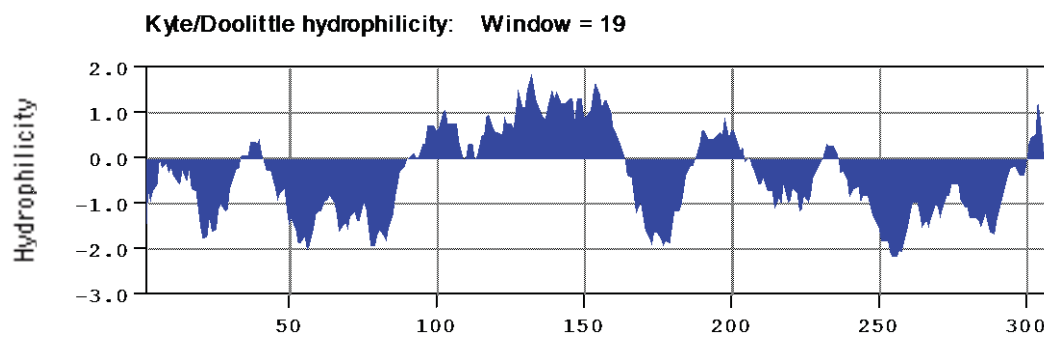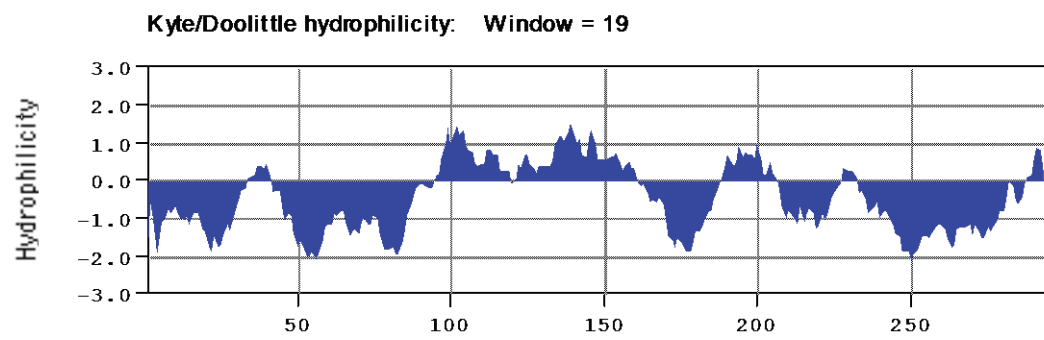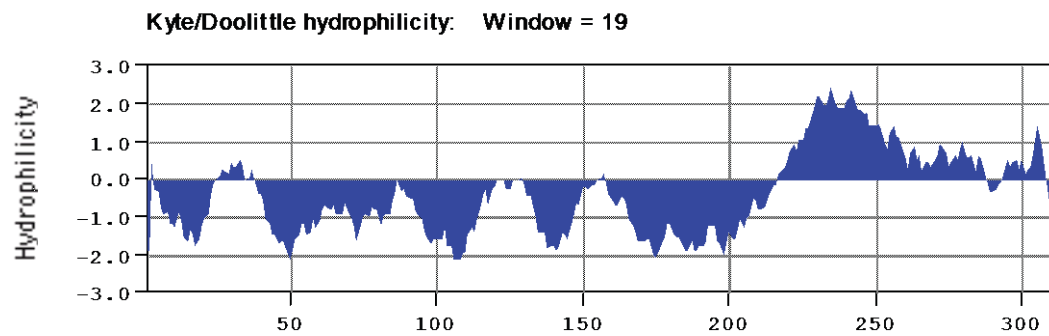
YPL264c
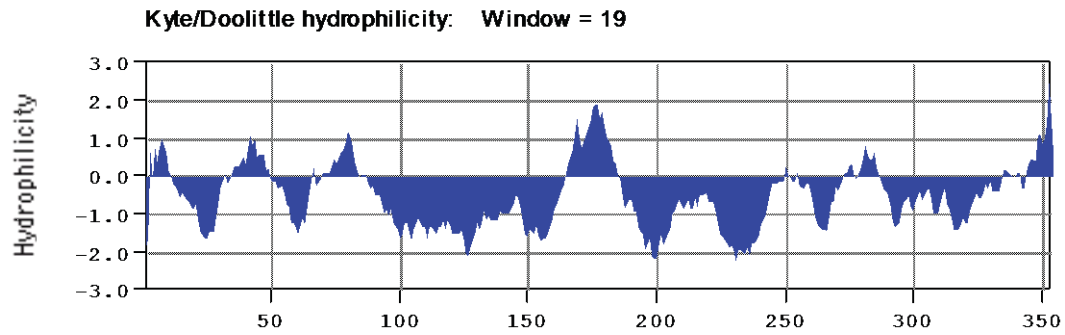
YGR149w
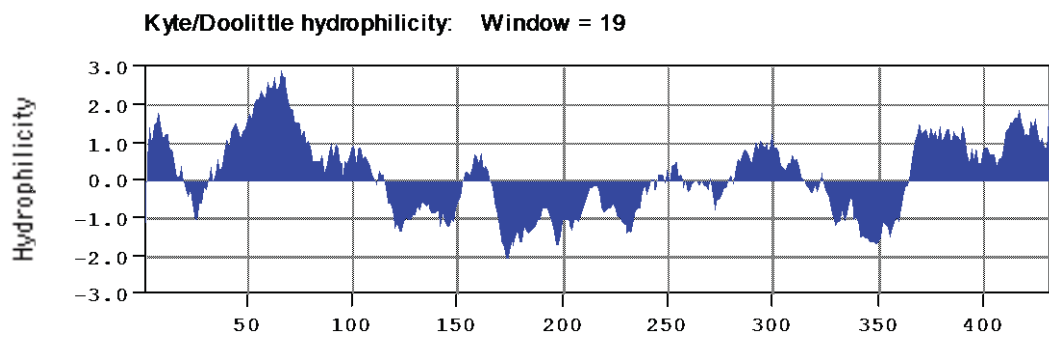
YFL034w
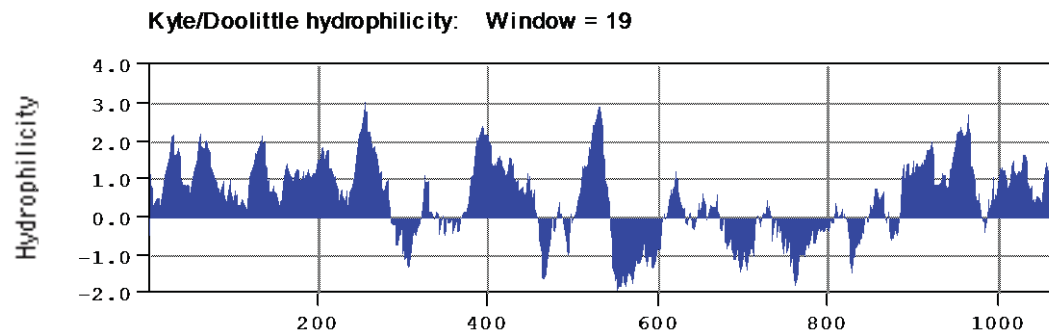
Figure 3-1: Hydrophillicity plots of candidate viNSCCs

Protein sequences of viNSCC candidate proteins were retrieved from the SGD and hydropathy plots generated using Macvector v 10.0 (MacVector Inc., NC, USA) using the Kyte / Doolittle protocol with an amino acid window size of 19. Regions which are hydrophobic have a negative value.

4. Identification of a protein family with voltage-dependent non-selective cation channel activity

4.1. Introduction

Voltage insensitive Non-Selective Cation Channels (viNSCCs) have been observed in many living systems including yeast (Bihler, 1998), plants (Demidchik, 2002a) and animals (Siemen, 1993). This class of cation channel is described as having relatively low discrimination between mono-valent cations (eg. Na^+, K^+, NH_4^+ Li^+ etc) and being inhibited by divalent cations, such as Ca^{2+} and Mg^{2+} (Siemen, 1993; Tyerman et al., 1995; Bihler, 1998, 2002; Demidchik, 2002a). Voltage insensitivity is not a strictly applied term as a protein showing some sensitivity to voltage may still be considered within the viNSCC class (Davenport and Tester, 2000). viNSCCs are thought to be strong candidates for low affinity cation fluxes in biological systems (Demidchik, 2002a; Britto, 2006). Such cation fluxes include the rapid flow of Na^+ observed in plants exposed to saline soil conditions (Essah et al., 2003) and the flux of NH_4^+ into plant roots at high external $[NH_4^+]$ (Tyerman et al., 1995; Britto, 2006).

4.1.1. PQ loop repeat proteins as putative viNSCCs in *Saccharomyces cerevisiae*

A *Saccharomyces cerevisiae* based assay was developed to search for putative viNSCCs (Chapter 2). This screen was dependent on the flux of the NH_4^+ analogue methylammonium (MA^+) through the viNSCC, producing a toxicity phenotype in yeast. By altering external Ca^{2+} concentration and the pH of the growth media, phenotypes that seem to be the result of functional viNSCCs were observed. This screen did not successfully identify a viNSCC. The high throughput methodology of this screen may have been a factor in the failure to find a candidate. It is possible that the phenotypic changes induced by the over-expression of candidate viNSCCs is too

subtle to be observed in such a screen. Another possibility is that the parameters of the screen, namely Ca^{2+} concentration and pH differences, may not have been deterministic enough to reveal a candidate amongst the vast biochemical changes when altering these conditions.

An *in silico* based screen was thus employed to help select candidates. These candidates were subjected to the previous *Saccharomyces cerevisiae* based assay under more stringent conditions than the large-scale screen (Figure 4-1). This screen identified two proteins of the PQ loop repeat class that showed the phenotypic response expected of viNSCCs over-expressed in yeast. Radioisotope cation trace experiments were then performed to confirm these proteins behave as putative viNSCCs.

4.2. Results

4.2.1. MA$^+$ toxicity based screen for putative viNSCC proteins

Five of the seven candidate viNSCCs identified in Chapter 3 were selected from the Open Biosystems Yeast ORF Collection (Open Biosystems) and over-expressed in the *S. cerevisiae* strain 31019b, which is deficient in all three high-affinity ammonium transport proteins ($\Delta mep1\Delta mep2\Delta mep3$) (Marini et al., 1997). This strain is mainly resistant to MA$^+$ except when external Ca^{2+} is reduced or when the media is prepared at higher pH levels (7 vs. 6.5) (Figure 2-1). A dilution series of transformed 31019b cells were spotted onto 100 mM MA$^+$ yeast media with high (10 mM) or low (0.2 mM) Ca^{2+} at either pH 6.5 or 7 (Figure 4-1 A, B, C and D). Across this range of treatments, three of the candidates (YDR352w, YOL092c and YFL034w) displayed a phenotype that could be a result of MA$^+$ accumulation due to the action of the over-expression of a viNSCC (Figure 4-1; B, C and D). MA$^+$ influx was confirmed by measuring ^{14}C labelled MA$^+$ accumulation over a period of 20 minutes (Figure 4-1; B, C and D). Over-expression of the remaining proteins, YGR149w, YPL264c and YOR291w, gave a phenotype no different to cells transformed with empty vector (data not shown) and were therefore no longer considered candidates. MA$^+$ sensitivity was induced on media containing 100 mM MA$^+$ by the expression of YDR352w and YOL092c. This was reduced through either lowering the pH to 6.5 and / or elevating Ca^{2+} concentrations from 0.2 mM to 10 mM (Figure 4-1; B and C). Cells expressing YPL034w were less sensitive to MA$^+$ than those expressing YDR352w or YOL092w (Figure 4-1; D). Cells over-expressing YFL034w were not rendered more sensitive to MA$^+$ toxicity when the Ca^{2+} concentration was lowered from 10 mM to 0.2 mM.

When media pH was raised from 6.5 to pH 7.0, there was a slight increase in MA$^+$ sensitivity (Figure 4-1; D). ^{14}C labelled MA$^+$ flux did not show a significant increase in MA$^+$ accumulation when compared to the empty vector control (Figure 4-1; D). YFL034w was subsequently discarded as a candidate viNSCC.

4.2.2.   MA$^+$ flux into *Saccharomyces cerevisiae* over expressing YDR352w or YOL092w

YDR352w and YOL092w were selected based upon their ability to impart a toxicity phenotype in cells of the *S. cerevisiae* strain 31019b in the presence of high MA$^+$ concentrations (Figure 4-1; B and C). Unfortunately, the original Open Biosystems cDNA collection from which YDR352w and YOL092w were isolated contained a C-terminal HA tag (Figure 4-2). To reduce the chance the HA-Tag may influence protein activity and observed phenotypes, these proteins were subsequently cloned from the genomic DNA of 31019b. Gateway adapted primers (Figure 4-3) were then designed and each gene amplified and cloned into pDONR222 (Invitrogen). They were then recombined into the yeast expression vector pYES3-DEST (M. Shelden, unpublished) (Figure 4-4) and transformed back into 31019b. The original plate screens were then repeated which fortunately produced identical phenotypes to those with containing the HA tag (data not shown).

To further characterise MA$^+$ influx by native YDR352w and YOL092w, the uptake of 50 mM ^{14}C labelled MA$^+$ was measured in 31019b. Cells over expressing either YDR352w or YOL092w enhanced MA$^+$ accumulation over time when compared to an empty vector control (Figure 4-5). Uptake followed an arithmetic regression and had an accumulation rate of 2.5 – 3 times that of the empty vector control at 30 minutes (Figure 4-5). Concentration-dependent MA$^+$ flux through these proteins was

also examined (Figure 4-6). MA^+ uptake followed an unsaturable profile significantly greater than that of the empty vector control. Cells over expressing YDR352w show a greater MA^+ flux capacity than those over expressing YOL092w, although they both show significant LATS activity relative to the empty vector control. Data for YOL092w and YDR352w are significantly different from each other only at the 30-minute time point (Figure 4-6).

4.2.3. Na^+ flux into *Saccharomyces cerevisiae* over expressing YDR352w or YOL092w

Na^+ accumulation of cells over expressing either YDR352w or YOL092w was investigated using ^{22}Na flux analysis (Figure 4-7). Na^+ accumulation was significantly greater in cells over expressing YDR352w and YOL092w when compared to empty vector control cells. The magnitude of this difference was not as large as for experiments using ^{14}C labelled MA and the data contained a greater degree of variance.

4.3.   Discussion

Two putative yeast viNSCCs, YDR352w and YOL092w, were identified which induced a MA-associated toxic phenotype when over expressed in the NH_4^+ transport deficient yeast strain 31019b (Figure 4-1). Four other candidates were discarded as potential viNSCCs, three (YDR149w, YPL264c and YOR291w) for showing no difference in MA^+ sensitivity when compared against the empty vector control. The fourth candidate, YFL034w, was not pursued further for not having significantly greater ^{14}C labelled MA^+ uptake compared to the control. Using ^{14}C labelled MA^+ or ^{22}Na labelled NaCl, the transport properties of YDR352w and YOL092w were further characterised. ^{14}C-MA uptake showed a consistently higher rate of MA^+ uptake in the LATS range for both YDR352w and YOL092w compared to the empty vector control when measured over a 30-minute period (Figure 4-5). A concentration profile showed these proteins exhibit unsaturable MA^+ uptake kinetics well above the LATS range of yeast of approximately 5 - 8 mM MA^+ (Figure 4-6). ^{22}Na flux experiments mirrored the MA flux data by showing an increased rate of Na^+ influx into cells over expressing YDR352w or YOL092w compared to empty vector transformed control cells (Figure 4-7).

The increased capacity of cation accumulation in cells expressing YDR352w and YOL092w is consistent with that expected for an over expressed viNSCC. Accumulation at both 50 mM MA^+ and 50 mM Na^+ was greater than that of the empty vector control. Net flux becomes significantly different from the empty vector control after 5 minutes for a 50 mM MA^+ buffer (Figure 4-5) or after 15 minutes with a 50 mM NaCl buffer (Figure 4-7). Similarly, differences between the empty vector

control and those expressing candidate proteins in the MA^+ concentration profiles was only obtained at 25 mM for YDR352w and 50 mM for YOL092w (Figure 4-6). When in a 50 mM NaCl buffer, Na^+ flux was not significantly different between YDR352w and YOL092w.

Although there was a significant response in MA^+ and Na^+ uptake with the over expression of YDR352W and YOL092w, it was evident there was uptake of these two ions in the empty vector-transformed yeast. It would appear native yeast proteins that facilitate either MA^+ or Na^+ flux or both are active in this strain. This could include both the native YDR352w and YOL092w proteins, most likely still active in 31019b, as well as other proteins of the PQ loop repeat class of proteins. It is also prudent to be careful of the assumption that the accumulation of these cations is a result of increased influx catalysed by the over-expression of these proteins. Whereas increased influx through these proteins is an obvious possibility, there are other possible explanations, such as decreased efflux of Na^+ / MA^+ from cells expressing these candidate proteins. Further flux characterisation of these a *Xenopus laevis* oocyte expression system do, however, support increased flux, in particular influx, through the expression of these proteins (Chapter 5).

Interestingly, total MA^+ accumulation was visibly lower than total Na^+ accumulation. This is most likely related to the reduced MA^+ permeability associated with the loss of the three native Meps which have been deleted from 31019b (Marini et al., 1997). As viNSCCs flux MA^+ and Na^+ with similar kinetics (Davenport, 1998), Na^+ accumulation could be expected to be similar to that of MA^+. The observed reduction of relative Na^+ flux compared to that seen for MA^+ is probably due to the action of

native yeast Na$^+$ transporters. Native proteins capable of effluxing Na$^+$ from yeast have been described (Haro et al., 1991; Prior et al., 1996) and are presumably active in the strain used for this flux experiment. These proteins will act to alter Na$^+$ accumulation in the yeast throughout these experiments, masking the overall influence the over-expression of YDR352w or YOL092w has on Na$^+$ accumulation. There is also the possibility that cell-wall-adsorbed tracer may have significant impact on background accumulation data observed. As the empty vector controls used in these experiments are exposed to the same conditions as those expressing the candidate genes, they can be considered to be the sum total of the effect of native protein-based tracer accumulation and tracer adsorption to the cell wall.

To improve the determination of these flux properties in yeast, constructs have been made to knock out YDR352w, YOL092w and a third PQ-loop homolog YBR147w in the 31019b background (data not shown). These strains will be used in future patch clamp and growth phenotype experiments to further characterise proteins from this class.

4.4.   Materials and Methods

4.4.1.   Heterologous expression of PQ loop repeat proteins in a *S. cerevisiae* expression system.

4.4.1.1.   Growth phenotypes on solid media of *S. cerevisiae* strain 31019b over-expressing candidate genes.

Vectors with candidate genes were sourced from the Open Biosystems Yeast ORF Collection. They consist of individual yeast ORFs cloned into the vector BG1805 (Figure 4-2). Each ORF is expressed under the high expression Gal1 promoter and has had its stop codon removed to incorporate a C-terminal HA protein tag (Gelperin, 2005). Each clone was transformed into 31019b (Marini et al., 1997) using the lithium acetate / poly ethylene glycol method (Gietz, 1995) and transformants selected on YNB minimal media. Transformed cells were individually grown overnight in liquid yeast nutrient base (BD biosciences, San Jose, USA; 0.67% (w/v), D-glucose 2% (w/v) pH 6.5) to late log phase. Cells were pelleted and washed twice in sterile milliQ water and re-suspended to OD_{600} of 0.3. Cultures were serially diluted to a final OD_{600} of 0.003 and 5μL of each dilution placed on solid yeast minimal media (Grenson, 1966) with 100 mM MA^+, 0.1% (w/v) L-proline , 2% (w/v) D-galactose at a pH of 7.0 with either 0.2 mM Ca^{2+} or 10 mM Ca^{2+}. Plates were incubated at 28°C for 5 – 7 days and growth phenotypes monitored.

4.4.1.1.1. Cloning of YDR352w and YOL092w into the yeast expression vector pYES3-Dest

YDR352w and YOL092w were cloned from genomic DNA of the strain 31019b using primers designed to flank the gene with *att*B1 and *att*B2 sequences. These were then incorporated into the vector pDONR222 (Invitrogen) via the BP reaction. Positive clones, determined by directional restriction enzyme digests, were then used to insert the gene into pYES3-Dest (M. Shelden, unpublished results, Figure 4-4). Positive clones were transformed into the *S. cerevisiae* strain 31019b using the lithium acetate / poly ethylene glycol method (Gietz, 1995) and transformants selected on YNB minimal media (- URA).

4.4.1.2. ^{14}C Labelled MA flux and ^{22}Na labelled Na$^+$ flux analysis

Cells were grown to saturation in liquid YNB supplemented with 2% (w/v) D-glucose, harvested via centrifugation at 4000 x *g* for 4 minutes and used to inoculate a modified minimal liquid media (Grenson, 1966) at pH 6.5 with 0.1% (w/v) L-proline and 2% (w/v) D-galactose to OD$_{600}$ = 0.1. Cells were incubated overnight at 28°C shaking at 200 rpm and harvested at OD$_{600}$ = 0.4 – 0.7 by centrifugation at 4000 x *g*, washed twice in MilliQ H$_2$O and resuspended in 20 mM K$_2$HPO$_4$ / KH$_2$PO$_4$ buffer pH 6.5 with 2% (w/v) D-galactose to give a final OD$_{600}$ ~ 4 - 6. A stock solution of 1 M MACl or NaCl was added to a 20 mM K$_2$HPO$_4$ / KH$_2$PO$_4$ buffer (pH 7.0) to the concentration required and labelled with either ^{14}C-MA (Amersham) or ^{22}Na (Amersham). This was added to an equal volume of resuspended cells at T = 0 and were shaken continuously throughout the flux experiment. At the specified time, samples were removed, cells collected using a 0.45 ⌠m nitrocellulose filter (Millipore)

and washed with 10 ml of ice-cold 20 mM K_2HPO_4 / KH_2PO_4 buffer to cease MA^+ or Na^+ flux. This wash step, combined with the use of an empty vector control, will minimise the impact of cell-wall-adsorbed tracer. Membranes were collected, placed in a 7 ml scintillation vial (Sarstedt) and 4 ml scintillation fluid (Perkin Elmer) was added. Samples were counted in a liquid scintillation counter (Packard). Counts were converted into equivalent amount of MA^+ or Na^+ and samples were normalised against total protein, derived from a modified Lowry method (Peterson, 1977).
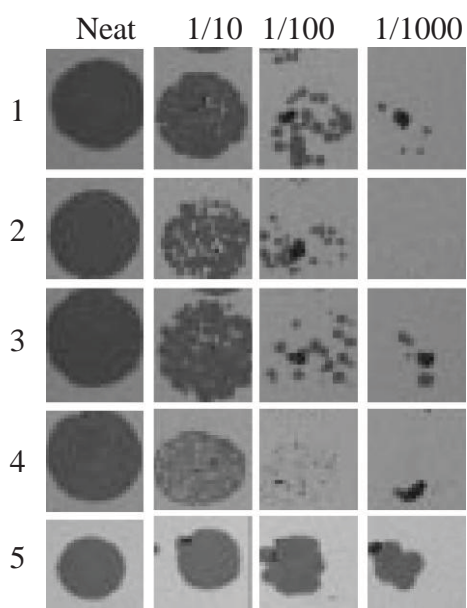
Figure 4-1, A: Variation in MA$^+$ sensitivity in *S. cerevisiae* strain 31019b transformed with the empty pYES3 vector

S. cerevisiae strain 31019b was transformed with empty pYES3 vector and cells were grown and plated as described (section 4.3.1.1). Minimal media (Grenson, 1966) was supplemented with 0.1 % L-proline, 100 mM MA$^+$ and 2% D-galactose with Ca^{2+} and pH modulated as follows - 1) 10 mM Ca^{2+} pH 6.5; 2) 0.2 mM Ca^{2+} pH 6.5; 3) 10 mM Ca^{2+} pH 7.0, 4) 0.2 mM Ca^{2+} pH 7.0; 5) YNB + 2% D-glucose as a loading control.
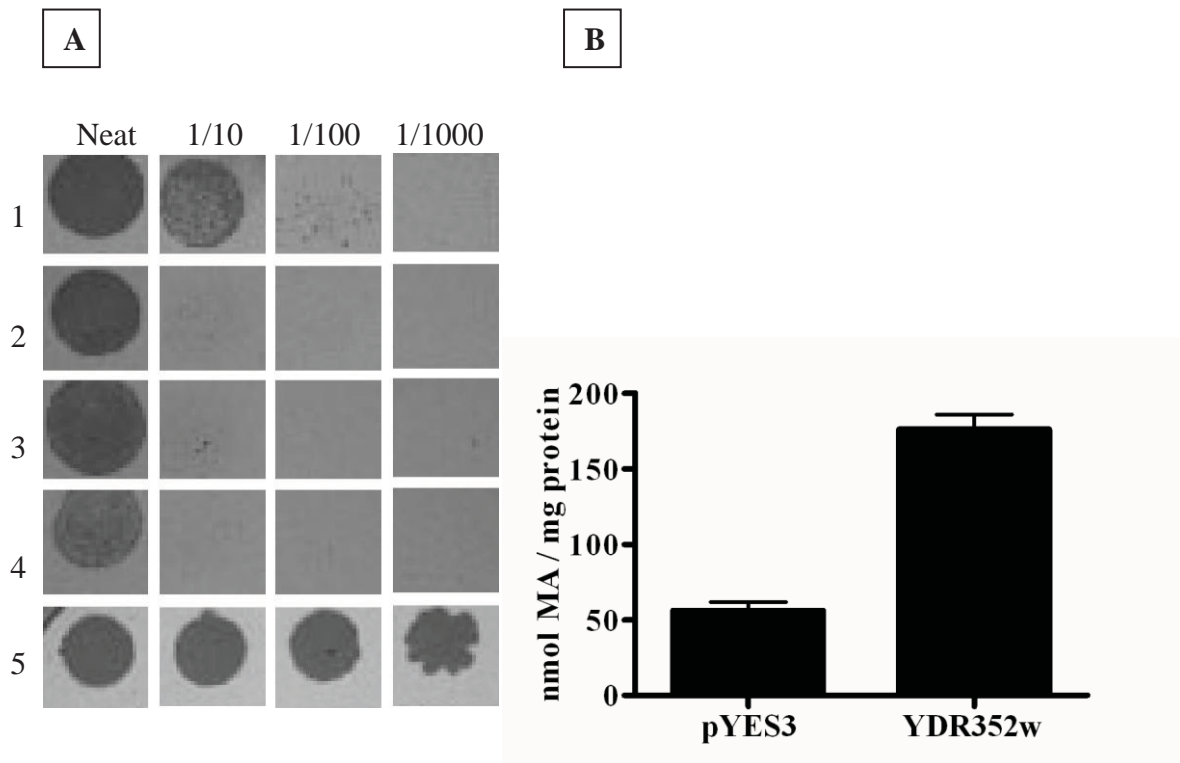
Figure 4-1, B: MA$^+$ induced toxicity and uptake in *S. cerevisiae* strain 31019b transformed with the YDR352w in the galactose inducible vector BG1805.

S. cerevisiae strain 31019b was transformed with the yeast expression vector BG1805 containing YDR352w. (A) Cells were grown and plated as described (section 4.4.1.1). Minimal media (Grenson, 1966) was supplemented with 0.1 % L-proline, 100 mM MA$^+$ and 2% D-galactose with Ca^{2+} and pH modulated as follows- 1: 10 mM Ca^{2+} pH 6.5; 2) 0.2 mM Ca^{2+} pH 6.5; 3) 10 mM Ca^{2+} pH 7.0, 4) 0.2 mM Ca^{2+} pH 7.0; 5) YNB + 2% D-glucose as a loading control. (B) Transformed 31019b cells were incubated in reaction buffer containing 0.5 mM ^{14}C-MA, 20 mM K$_2$HPO$_4$ / KH$_2$PO$_4$ and 2% D-galactose at pH 7.0. Net uptake of ^{14}C MA was measured in harvested cells using a scintillation counter after 20 minutes incubation. Data presented is mean ± SE (n=10).
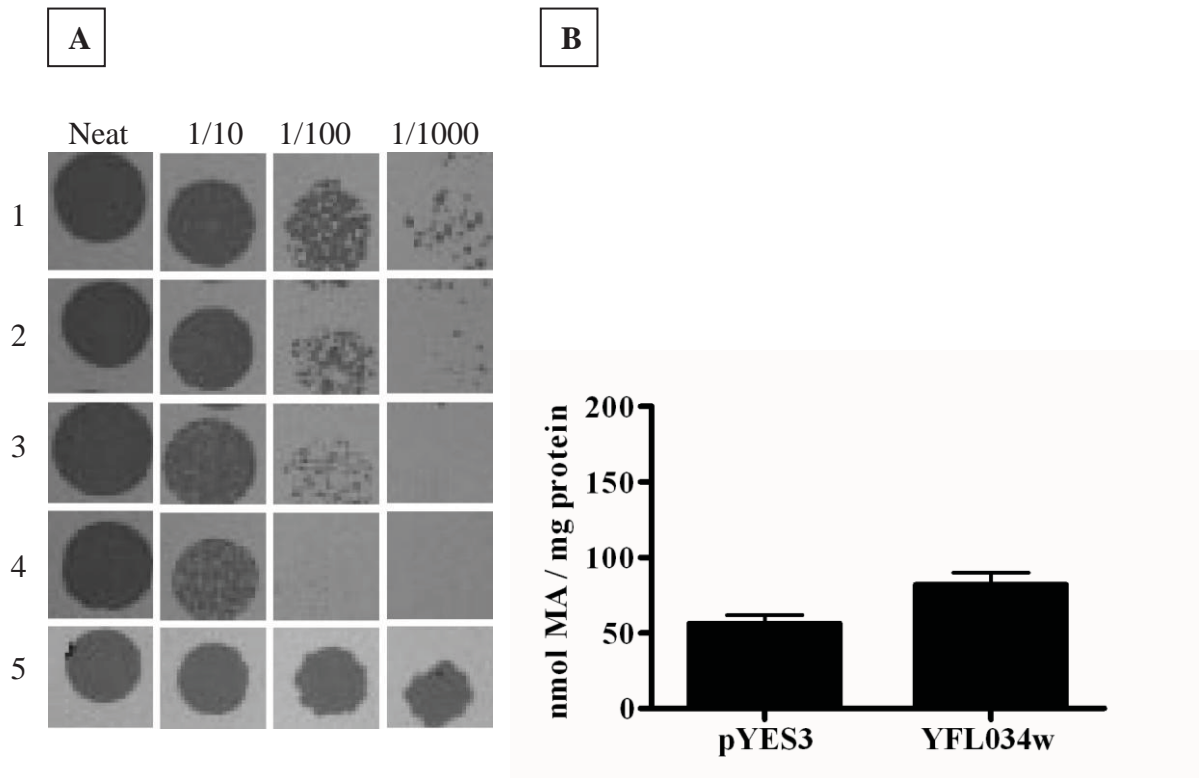
Figure 4-1, C: MA$^+$ toxicity and uptake in *S. cerevisiae* strain 31019b transformed with YOL092w in the galactose inducible vector BG1805.

S. cerevisiae strain 31019b was transformed with the yeast expression vector BG1805 containing YOL092w. (A) Cells were grown and plated as described (section 4.4.1.1). Minimal media (Grenson, 1966) was supplemented with 0.1 % L-proline, 100 mM MA$^+$ and 2% D-galactose with Ca^{2+} and pH modulated as follows- 1: 10 mM Ca^{2+} pH 6.5; 2) 0.2 mM Ca^{2+} pH 6.5; 3) 10 mM Ca^{2+} pH 7.0, 4) 0.2 mM Ca^{2+} pH 7.0; 5) YNB + 2% D-glucose as a loading control. (B) Transformed 31019b cells were incubated in reaction buffer containing 0.5 mM ^{14}C-MA, 20 mM K$_2$HPO$_4$ / KH$_2$PO$_4$ and 2% D-galactose at pH 7.0. Net uptake of ^{14}C MA was measured in harvested cells using a scintillation counter after 20 minutes incubation. Data presented is mean $\pm$ SE (n=10).
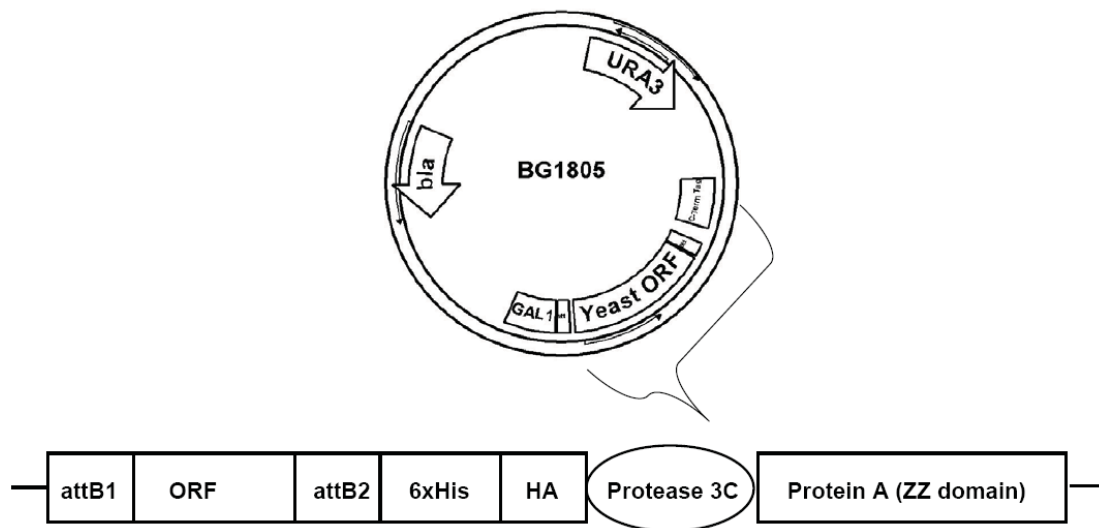
Figure 4-1, D: MA$^+$ toxicity and uptake in *S. cerevisiae* strain 31019b transformed with YFL034w in the galactose inducible vector BG1805.

S. cerevisiae strain 31019b was transformed with the yeast expression vector BG1805 containing YFL034w. (A) Cells were grown and plated as described (section 4.4.1.1). Minimal media (Grenson, 1966) was supplemented with 0.1 % L-proline, 100 mM MA$^+$ and 2% D-galactose with Ca^{2+} and pH modulated as follows- 1: 10 mM Ca^{2+} pH 6.5; 2) 0.2 mM Ca^{2+} pH 6.5; 3) 10 mM Ca^{2+} pH 7.0, 4) 0.2 mM Ca^{2+} pH 7.0; 5) YNB + 2% D-glucose as a loading control. (B) Transformed 31019b cells were incubated in reaction buffer containing 0.5 mM ^{14}C-MA, 20 mM K$_2$HPO$_4$ / KH$_2$PO$_4$ and 2% D-galactose at pH 7.0. Net uptake of ^{14}C MA was measured in harvested cells using a scintillation counter after 20 minutes incubation. Data presented is mean ± SE (n=5).

Figure 4-2: Vector map of BG1805

Candidates genes were supplied in the yeast expression vector BG1805 (Open Biosystems) (Gelperin, 2005). Each ORF has a stop codon mutation that allows the tagging of each protein with the construct illustrated.

YDR352w Fwd:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGTTCGTTGTTATCGGTGCT

YDR352wRev:

GGGGACCACTTTGTACAAGAAAGCTGGGTCAATGGTAGTACTCCGTAGGGAAA

YOL092w Fwd:

GGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGCTACCACATCGCTCGAC

YOL092w Rev:

GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGAGGTCAAAAATATGTTAAATAAA

Figure 4-3: Primers used in the amplification of Gateway (Invitrogen) adapted primers for full-length clones of YDR352w and YOL092w from yeast genomic DNA.
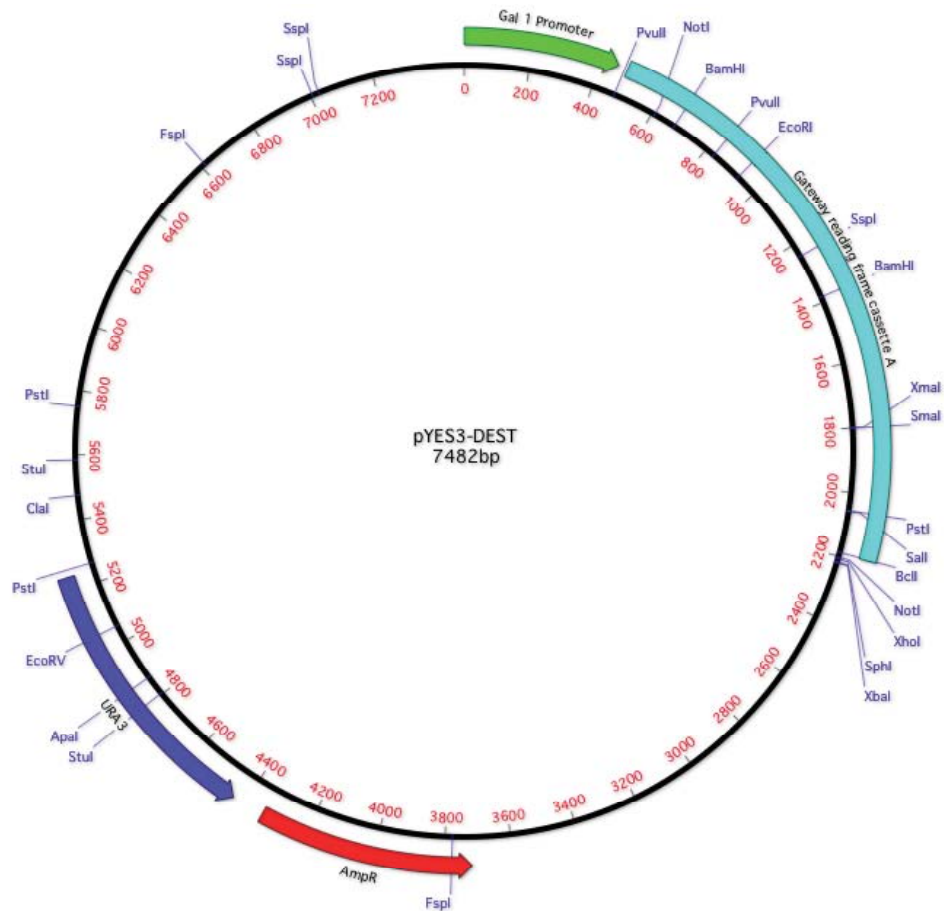
Figure 4-4: The yeast expression vector pYES3-DEST

pYES3 {Smith, 1995 #185} was modified by M. Shelden (unpublished data) to incorporate an attR1 and attR2 flanked gateway reading frame. This vector was subsequently used in the expression of genes in *S. cerevisiae*.
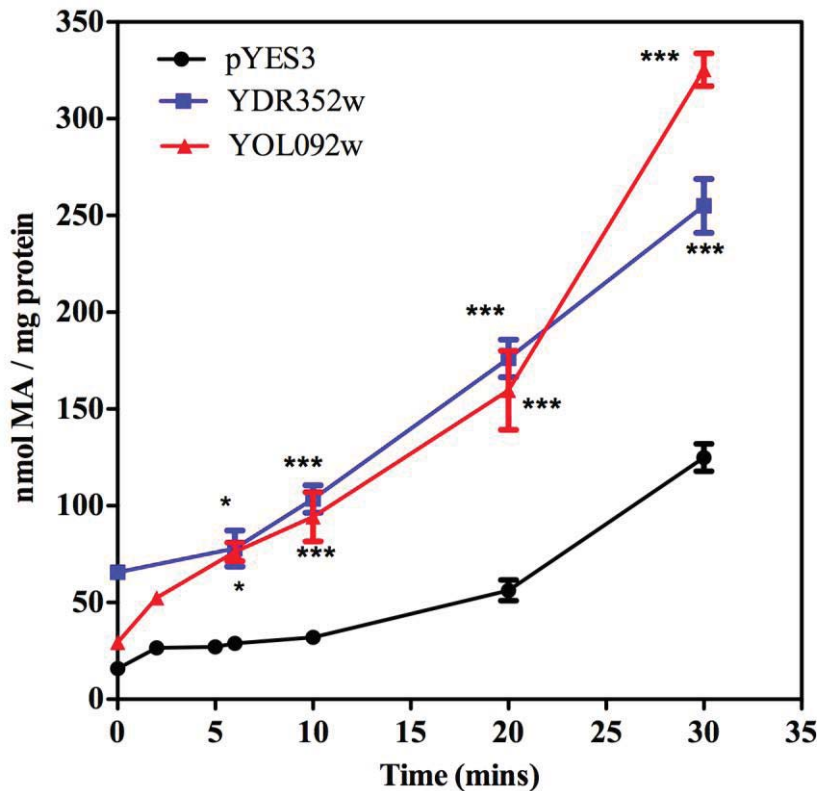
Figure 4-5: Time dependent uptake of ^{14}C-MA by *S. cerevisiae* strain 31019b expressing YDR352w, YOL092w or the empty vector pYES3 control.

S. cerevisiae strain 31019b was transformed with pYES3-DEST containing either YDR352w, YOL092w or no insert. Cells were grown as described in the methods of this chapter and re-suspended after washing in a 20 mM K_2HPO_4 / KH_2PO_4 buffer at pH 7.0. ^{14}C labelled MA^+ (50 mM) was added at T = 0 and cells collected by filtration through a 0.45 μm membrane. MA^+ content was determined relative to total cell protein. Data presented is the mean ± SE (n=5) where significance was determined through two-way ANOVA. Data points with *** are significantly different from controls for $P < 0.001$ and data points with * are significantly different from controls for $P < 0.05$. YDR352w and YOL092w differ significantly only after 30 minutes of flux.
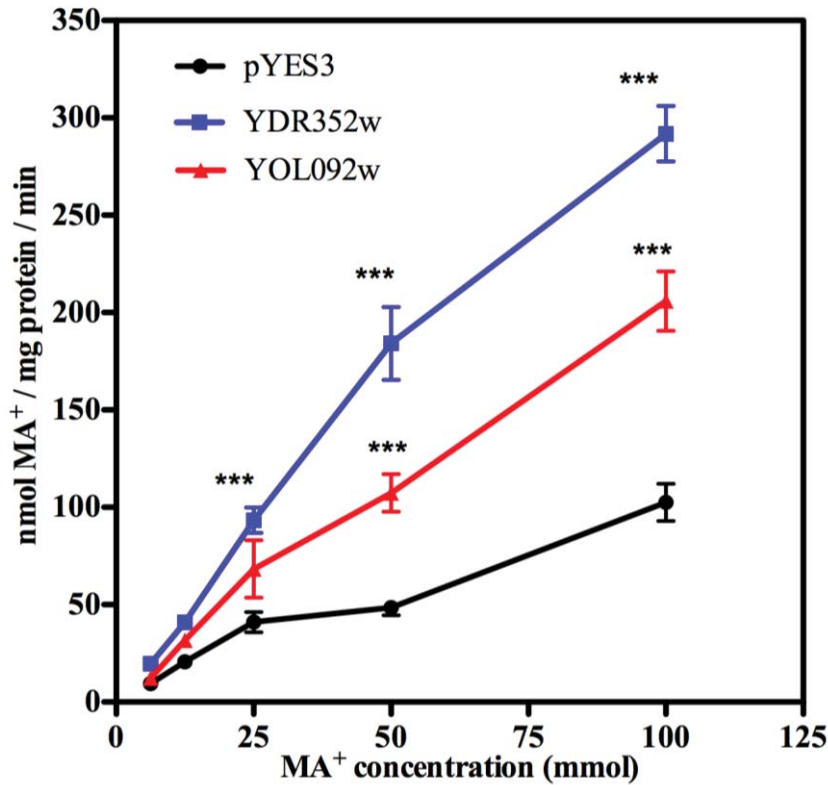
Figure 4-6: Concentration-dependent ^{14}C-MA uptake by YDR352w and YOL092w.

S. cerevisiae strain 31019b was transformed with pYES3 containing either YDR352w, YOL092w or no insert. Cells were grown as described in the methods section of this chapter and resuspended in a 20 mM K_2HPO_4 / KH_2PO_4 buffer at pH 7.0. ^{14}C-MA was added at various concentrations and cells collected 5 minutes later by filtration through a 0.45 μm membrane. MA^+ content was determined relative to total cell protein. Data presented is the mean rate $\pm$ SE (n=6). Data points with *** are significantly different from controls (P < 0.05). YDR352w and YOL092w are significantly different to each other (P < 0.001) at 50 mM and 100 mM concentrations.
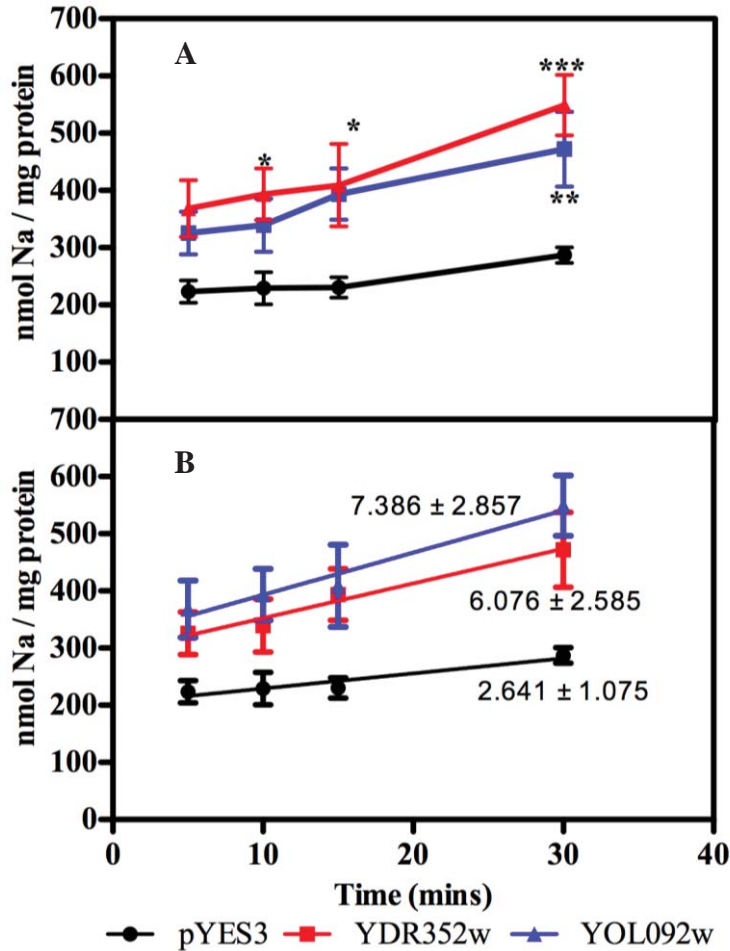
Figure 4-7: Time dependent uptake of ^{22}Na$^+$ by *S. cerevisiae* strain 31019b expressing YDR352w, YOL092w or the empty vector pYES3 control.

S. cerevisiae strain 31019b was transformed with pYES3 containing either YDR352w, YOL092w or no insert. Cells were prepared and used as described in the methods of this chapter, using 50 mM ^{22}Na labelled NaCl added at T = 0. Data presented is the mean rate ± SE (n=6). Data points with ***, ** and * are significantly different from controls for $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively. A shows total Na flux into cells and B is the linear regression of data over this time period with annotations showing the slope of each line. The higher value of slope indicates an increased in Na$^+$ flux as a result of over expressing of YDR352w or YOL092w.